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HYPERTROPHY OF THE PHARYNGEAL OR LUSCHKA'S TONSIL

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Hypertrophy of the pharyngeal or Luschka's tonsil, by some authors termed adenoid growths, consists of an abnormal enlargement of the glandular tissues usually found in the vault and walls of the pharynx, characterized by obstruction of nasal respiration, catarrhal symptoms and in many cases partial deafness, with alterations in the voice partially due to obstruction of the resonant chamber in the posterior nares, and partially to changes in the larynx caused by mouth-breathing. There is also more or less deterioration of the general health, and frequently deformity of the chest, in many instances mental debility and imperfect physical development are very pronounced. Attention was first directed to this condition by Meyer, of Copenhagen, in 1860, and later by Loewenberg, of Paris, who reported three cases in 1865. The same author subsequently published a complete *résumé* of the subject in 1879. A thorough discussion of the subject was brought out

Meyer's paper at the International Medical Congress in London in 1881, since which time so many articles have been written upon the subject that it has been worn almost threadbare. Nevertheless it appears to me that an analysis of a considerable number of cases occurring in private practice may show some new items of interest concerning the condition, or at least aid in settling some of the uncertain points in its clinical history.

Withstanding the many cases that have been reported by laryngologists, the disease is not so common as it could at first appear. I find among my records of private patients since 1891, histories of between 11,000 and 12,000 persons of whom about 1 per cent or in even numbers 10,000, came to me on account of some disease of the chest, throat or nasal cavity. Of these, 203 of in even numbers, 200, equaling only 2 per cent of the whole, had hypertrophy of the pharyngeal tonsil. It is impossible to determine the relative frequency of diseases of the chest, throat and nose, but is only 2 per cent of these special cases suffer from enlargement of this gland, it is clear that the percentage must be infinitely smaller when all classes of disease are considered.

I find in 10,000 consecutive cases of diseases of the chest, throat and nasal cavities, 200 cases of hypertrophy of the pharyngeal tonsil. Of these 65 per cent were residents of the city, and 35 per cent came from the country or smaller towns. As only 27 per cent of the whole number of my private patients

within the last decade have come to me from out of the city it would appear upon the face of these statistics that the disease is about 50 per cent more frequent in the country than among residents of the city. This does not agree with my deductions when considering the influence of sex, where I state that because of their living more out of doors boys seem less liable to the disease than girls. Possibly the disparity in this respect is due to the relatively larger number of city physicians who would treat this affection, thus diminishing the number of such patients who would consult any one specialist.

Out of the 200 cases of hypertrophy of Luschka's tonsil that have consulted me, in some the condition was of so little importance that it had not attracted the patient's attention, and was only discovered incidentally in making the ordinary examination, in many others I was consulted merely for diagnosis, and in still others for various causes the history has been imperfectly taken so that the records are worthless for purposes of study. Excluding most of the foregoing I have selected 100 cases as nearly consecutive as possible, not from any special feature which the case might present, but simply because of the completeness of the history, and I have excluded a considerable number of cases similar to these, not from any peculiarity of the history, but because it is more convenient to deal with an even hundred. These cases therefore, ought not to be considered as selected cases in the ordinary acceptance of this term, though it is probable that patients presenting the most pronounced symptoms were most carefully interrogated by my assistants who took the histories.

In my further consideration of this subject I shall confine myself to a study of these 100 cases which I think fairly and honestly represent the cases of well developed hypertrophy of the pharyngeal tonsil who would present themselves to a physician in private practice in this country.

The affection nearly always develops in infancy or early childhood. From the ages of the patients recorded it appears that the disease is most frequent in children between the ages of 2 and 15, 54 per cent being observed between the second and eighth year and 37 per cent between the ninth and sixteenth. But the affection is also seen in older persons. Seven cases were found between the ages of 18 and 25, and one each at the ages of 26 and 37 respectively. These results differ widely from Meyer's, who observed less than 37 per cent under 10 years of age, and less than 62 per cent all told under 15. They differ even more widely from Bosworth's statistics which show less than 30 per cent under 15 years of age. The difference is probably to be explained by the fact that I have ignored cases in which the growth had not attained sufficient size to cause the patient inconvenience, or had atrophied to such an extent as to have become unimportant.

A more critical examination of the histories in the endeavor to ascertain when the symptoms first appeared, indicates that the disease was certainly developed in 90 per cent of the cases in infancy or very early childhood. Many of these cases were said to have had difficulty in breathing since birth, and it is probable that in some of these the growth was congenital. I doubt very much whether the disease is ever developed after puberty.

From my statistics the affection appears to be more frequent in girls than in boys, 41 per cent having been recorded as males and 59 per cent females. This is possibly due to the fact that boys being so much more out of doors than girls are apt to be more vigorous and consequently less liable to hypertrophy of the adenoid tissues.

In Meyer's cases a little over 51 per cent were males, but in Bosworth's cases only 36 per cent were males. When it is remembered that in Copenhagen and its vicinity, the girls live more as do the boys than in this country, and are consequently more

vigorous, my explanation of the influence of sex is partially substantiated and it appears probable that under the same hygienic conditions there

would be no difference in the frequency of this affection in the two sexes. Militating against the correctness of this view is the fact that Greville MacDonald and Sir Morell Mackenzie found the disease considerably more prevalent in males. This, however, may have been due to their considering many cases in which the gland had dwindled into insignificance, for it would appear reasonable to suppose that as puberty occurs at an earlier age in girls than in boys and as these glands usually atrophy at that period, the hypertrophy among girls would be noticed less frequently among them after the age of 11 or 12 than among boys of similar years. The fact that Woakes, also of London, found this affection about equally in the two sexes suggests that there must have been some radical difference in the method of analyzing the cases by different observers. Keeping in view the fact that 90 per cent of the cases I am considering were observed before the age of puberty, and that all of them were well marked, there seems little room for doubt that, in this locality, at least, the disease is more prevalent among girls than boys.

The anatomic and pathologic characteristics of this affection have been so thoroughly described by Meyer, Loewenberg, and subsequent writers that I will pass them by.

Etiology—In an affection which has generally existed for years before the physician is consulted, it is impossible to obtain any very accurate idea of the causation, but this analysis seems to prove that enlargement of Luschka's tonsil in the great majority of cases is due to the same causes as hypertrophy of the faucial tonsils. It is attributed by various authors to hereditary influences, to the stumous and rheumatic diathesis, to diphtheria and the exanthematous diseases, to catarrhal troubles and to frequent colds.

I find among my cases several that appear to be congenital, though in none of these could this be stated certainly. Ten per cent were apparently of hereditary origin as shown by the occurrence of two or more cases in the same family, 3 per cent appeared to follow immediately after measles, 4 per cent after scarlet fever, 1 per cent after diphtheria, 3 per cent were attributed by the parents, or

the patient, to pneumonia or influenza, and a large number, amounting to 16 per cent, were attributed to frequent colds, or to what is doubtless included in the classification of some authors as a catarrhal condition. In 63 per cent nothing could be discovered which appeared in any way to account for the origin of the disease. I find in the same connection that 35 per cent of these patients were subject to frequent diseases of the throat.

In 76 per cent one or both of the faucial tonsils were also enlarged in the same case, in 7 per cent one, and 69 per cent both. This coincident enlargement of the faucial tonsil occurs in such a large number of cases as to indicate that the two conditions are commonly dependent upon the same causes, and it leads us to suspect that many of the results formerly attributed to enlargement of the faucial tonsils, more especially affections of the eye and ear and alterations of the voice, were in reality due to enlargement of Luschka's tonsil. In the great majority of cases where any of these symptoms just referred to appear with enlargement of the faucial tonsils, excision of the latter will be found to have but little influence, unless the pharyngeal tonsil is also removed. Scrofulous affections or enlargements of glands in other parts of the body were noted in only 2 per cent of the cases.

Symptomatology—The symptoms which are most commonly noticed by the parents or friends are snoring, loud breathing at night, restlessness, sleep with bad dreams, mouth breathing and general debility. The most frequent objective symptoms are impairment of the voice, deafness, and partial loss of the sense of smell. The patient's strength as indicating his general condition is noted in 65 cases, in 80 per cent of which it was found to be good, in 18 per cent only fair, and in 2 per cent poor. The general debility apparently resulted in most of these cases from the imperfect aeration of the blood in consequence of obstructed respiration. Pains in various parts of the body were complained of in a few instances. The sense of sight was affected in 1 per cent of the cases, and the sense of smell in 11 per cent. Frequent headache was complained of in 27 per cent of the cases. Deafness is a common result of hypertrophy of Luschka's tonsil, the sense of hearing having been found impaired in 33 per cent of these cases. In only 38 cases has any reference been made to the patient's mental condition, but because of the frequency with which these patients appear to be dull, I am surprised to find that in only one case is it noted that there was any impairment of the mental condition, and in this case it is stated that the patient appeared to be idiotic, a condition which seemed to have had no relation to the obstruction of the nasopharynx. The condition of the voice is noted in six of five cases, in 30 per cent of these it is said to be normal, but in fifteen muffled, impeded or thick, in four nasal, and in fifteen hoarse or husky, showing that it was affected in 70 per cent of the cases where the condition of the voice was mentioned.

The condition of the respiration was noted in forty-six cases, in 60 per cent of which it was found to be unaffected, but in 40 per cent there was decided dyspnea. In only 8 per cent of these the dyspnea was said to have occurred at night or during sleep, while in the remaining 32 per cent the dyspnea was also noticed during the day.

More or less cough is a frequent symptom, with

regard to which I find notes in seventy three cases, of these 60 per cent had no cough, in 12 per cent it was described as slight or inconsiderable, in 12 per cent as hacking to clear the throat, and in 12 per cent as severe. In 6 per cent of these seventy-three cases the cough occurred especially at night, or in the early morning.

The condition of the nasal cavities is noted in eighty-six cases, in 30 per cent of which they were described as free or normal, in 12½ per cent they were obstructed by exostosis from the septum, and in 50 per cent by hypertrophy or swelling of the inferior turbated bodies. Excessive secretion or other catarrhal symptoms were also noted in 60 per cent of the cases. This would appear to sustain the theory advanced by MacDonald ("Diseases of the Throat and Nose," 1890), that a majority of cases of enlargement of Luschka's tonsil are due to obstruction of the nasal passages consequent upon rarification during inspiration of the air in the naso pharynx. However, as my records do not enable me to determine whether in these cases the obstruction of respiration preceded or followed the enlargement of Luschka's tonsil, the evidence for or against this proposition can not be considered conclusive, but there has always been a question in my mind whether the obstruction of the nasal cavities from swelling of the soft tissues in such cases was not the result rather than the cause of the hypertrophy of the pharyngeal tonsil.

The excessive discharge in nearly all of these cases is clearly the result of obstruction in the naso-pharynx, which independently of its influence on the congestion of the mucous membrane, prevents evaporation of the secretions and causes their collection in the nasal fossæ.

In many cases, enlargement of the pharyngeal tonsil may be suspected from the appearance of the follicles in the lower portion of the pharynx. I find the condition of the pharynx noted in eighty-one cases, in 25 per cent of which the follicles were found decidedly enlarged.

Deafness is noted in forty cases, the right ear was affected in twenty four, the left in twenty-one, but there seems to be no relation between deafness upon one side and enlargement of the corresponding faucial tonsil, indeed, deafness appears to have been present quite as frequently when the faucial tonsils were but little enlarged as where these latter organs were greatly hypertrophied; therefore, so far as these statistics go they would seem to substantiate the statement already made, that affections of the special senses formerly supposed to have resulted from hypertrophy of the tonsils, have doubtless in most cases been caused by enlargement of the pharyngeal tonsil. In twenty-two of these the deafness was entirely cured or greatly improved by the treatment.

The form of the chest walls has been noted in only fifteen cases, but it is fair to suppose that in a majority of the others it was in a normal condition, otherwise it would have attracted the parents' attention and have been mentioned in the course of the examination. In the records where the condition of the chest walls is referred to it was normal in twelve and more or less deformed in only three of the cases.

Diagnosis—The diagnosis in these cases was usually made by inspection of the posterior nares with the rhinoscope, aided sometimes by anterior rhinoscopy and the nasal probe, but in young or unmanageable children in whom the throat mirror could not be

properly used, it was verified by the finger passed into the naso pharynx.

Treatment—Many of the cases that have come under my observation were brought to me simply for diagnosis and suggestions for treatment, the patient subsequently having been placed under the care of some other physician. In some instances where the parents feared an operation, internal treatment has been recommended and carried out for a greater or less period of time, sometimes with advantage but in others with no effect. In still other instances surgical procedures have been carried out by other physicians, and I have been unable to obtain information of the results.

In the whole 200 cases, removal of the gland by operation was recommended in only 120 or 60 per cent, and in the 100 cases I am now considering it was recommended in only 90 per cent, and performed by me in only 76. In a considerable number of the patients upon whom I have operated, the records of treatment are very imperfect for the reason that speedily after the gland was removed the symptoms disappeared and the patient was considered well, so that I have heard nothing more of the case.

The records show that in fourteen cases internal treatment consisting of syrup of the iodid of iron or syrup of hydriodic acid, was suggested, and that in ninety cases surgical procedures were recommended. By consulting my records and the replies of parents or friends to my letter of inquiry, I find that the hypertrophied gland was removed in 76 of these 100 cases, that it was treated by local applications of chromic acid in 11 cases, but that the patient received internal treatment only in 13 cases. In fifty of the cases operated on, one or both of the faucial tonsils were also found more or less enlarged and in twenty-two cases they were removed at the time of the operation on Luschka's tonsil.

One of the most notable results of freeing the naso pharynx from this obstruction has been its effect upon the general condition. It is not uncommon for young children within six months after the operation to gain from 20 to 40 per cent in weight and correspondingly in vigor and endurance.

Although in very few cases was the mental condition noted when the record was first made, I find by inquiry of parents or friends of the patient that the mental condition has been improved in 12 per cent of cases in which an operation has been performed.

Deafness was noted in only forty cases and the effects of treatment upon the sense of hearing was recorded in only thirty seven. In 30 per cent of these there had been no improvement, in 4 per cent there has been slight improvement, and in 66 per cent the deafness has been greatly ameliorated or cured, the latter result being noted in 33 per cent of the cases.

In the whole number of cases the voice was noted to have been affected in forty five. In three of these no improvement was observed as a result of treatment, in some no subsequent history could be obtained, but in thirty five, or 77 per cent, great improvement has been observed.

Of these 100 cases 56 complained of catarrhal symptoms. Eighty-six per cent of these have been greatly improved by the treatment, though 14 per cent seem not to have been benefited. This appears to justify the opinion, already expressed, that the obstruction of the nasal passages is often

instead of the cause of hypertrophy of the pharyngeal tonsil

There is an undoubted tendency for hypertrophy of the pharyngeal tonsil to disappear by atrophy about the time of puberty, but an analysis of these cases, of which 9 per cent were over 16 years of age, shows that this fortunate result is not so common as generally supposed. There appears to be a common impression among laryngologists that unless such growths are removed by surgical measures the condition of the patient is almost sure to go from bad to worse, until the twelfth or fourteenth year, when atrophy usually begins. In twenty-seven of these cases, for various reasons no radical operation has been performed. Eleven of these were treated by applications of chromic acid through the nostril, and I find that eight of them were apparently cured by this treatment. In the remaining sixteen cases we have relied entirely upon internal treatment, consisting of the syrup of hydriodic acid or of the iodid of iron, the latter being given when anemia was apparent. I have been unable to obtain the subsequent history in four of these. In 50 per cent of the twelve whose histories are completed there appears to have been great improvement or complete recovery, apparently as a result of the treatment. Four others were somewhat improved from various causes, and two appeared to have made no gain. This goes to show that although the most brilliant and satisfactory results are obtained by surgical procedures, constitutional remedies are adequate to the removal of the disease in a considerable number of cases, but this should not be made an excuse for allowing the patients with well marked hypertrophy of this gland to go on from year to year in the hope that internal remedies or time may effect the cure, because in the meantime in very many cases, inflammation of the Eustachian tube and middle ear will have caused deafness, or the voice may have become permanently altered, while deprivation of the patient of the amount of oxygen to which he is normally entitled will have stunted his growth, impaired his constitution, and possibly will have left a permanent impress upon his mental faculties from which nothing can ever relieve him.

An analysis of these cases leads to the following conclusions:

Hypertrophy of Luschka's tonsil is comparatively infrequent, occurring in only 2 per cent of the patients with diseases of the chest, throat and nasal cavities, and probably in not more than one half of 1 per cent of all cases.

In this locality the disease is apparently 50 per cent more frequent in the country than in the city, and it occurs more frequently in girls than in boys.

The disease is observed in 90 per cent of the cases before the fifteenth year of age and is probably always developed in infancy or early childhood, some cases appearing to be congenital.

Ten per cent of the cases are apparently hereditary. Sixteen per cent may be attributed to frequent colds, but in 63 per cent no etiologic factor can be discovered.

In the great majority of cases the affection is apparently due to the same causes as enlargement of the faucial tonsils, and in 76 per cent of the cases the faucial tonsils are also hypertrophied.

In a considerable number of cases, amounting to 18 per cent the general health is materially injured by this affection.

The sense of smell is obtunded or lost in 11 per cent.

Hypertrophy of Luschka's tonsil is a frequent cause of headache, it being present in 27 per cent of the cases.

It is a frequent cause of deafness, the hearing being affected in one third of the cases. The deafness so caused may be benefited in a large majority (70 per cent) of cases by operative procedures, 66 per cent being greatly ameliorated and about half of these being completely cured.

The disease causes much alteration in the voice in about 70 per cent of the cases. In nearly eight-tenths of these the voice may be greatly improved as the result of treatment.

In about 40 per cent of the cases there is decided dyspnea, resulting from partial closure of the nasopharynx.

Forty per cent of these patients have more or less cough, in about one-third of which it is quite severe.

The pharyngeal follicles are enlarged in about one-fourth of all the cases. The nasal cavities are more or less closed by swelling or hypertrophy in 50 per cent of the cases, and 56 per cent complain of catarrhal symptoms, but in about nine-tenths of these the symptoms are removed by extirpation of the glandular tissue.

Although deterioration of the general health is not usually complained of, it is found as the result of operation that in many cases great improvement occurs immediately, the patient often gaining 30 per cent in weight within six months.

THE THROAT APPEARANCES IN MYXEDEMA

Read in the Section on Laryngology and Otology, at the Forty-fifth Annual Meeting of the American Medical Association, held at San Francisco June 5-8 1894.

BY H. HOLBROOK CURTIS, M.D.

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One year ago a patient presented herself at my office to have her throat examined, in order to undergo treatment for difficulty in speaking distinctly. Her history from my note book was as follows: Age 48, weight 190 pounds. Had five children. Menstruation stopped two years ago. Suddenly became very stout about ten years ago, after a prolonged and exhaustive experience with nursing a sick relative, at the same time undergoing intense mental agitation from other sources. Has had no perspiration for ten years. Water always very distasteful as a drink. Partakes sparingly of tea and coffee. Six years ago legs became glazed and waxy, the small hairs disappearing from same. Some edema of ankles but no pitting on pressure. Had been twice to Carlsbad for jaundice within seven years. Much brown pigmentation on abdomen. Had to stop playing piano some six years ago, because great weakness developed in her arms. Walking became very distasteful. Commenced at this time to become partially deaf. Could only hear in theater in the front rows. Eyes also commenced to appear small by reason of swelling of the lids. Speech became thick every afternoon at 5 o'clock, but without hoarseness. Often disinclined to talk and became sad and morose. Eighteen months ago throat symptoms became more marked. Often in talking a spasm would choke her. Patient likened it to a lump of the throat. Far back on the sides of the

the noticed d

ongue a marked stiffness was complained of, and the joint seemed thick and difficult to control. The end of her tongue was always "in the way." During this time patient always slept well. Pulse 72, temperature slightly subnormal. Patient was always cold, and very anemic. Ears presented a waxy appearance. More recently the gums were swollen and deficient in nutrition. The lips had recently become difficult to manage easily, they felt very thick and stiff. Was apt to say bore for more etc. The last two years mental symptoms had commenced to develop. Patient would frequently use the wrong word but always corrected it herself. It was very often a great effort to think quickly. A sudden surprise would make speech almost impossible. Coming into a room where there were strangers would confuse ideas and speech. Upon examination of the nose, the mucous membrane appeared bluish and pale, the turbinate bodies enlarged and very soft (inflated) making nasal respiration wellnigh impossible. The uvula was very thick, very pale with a yellowish tinge, and the pillars of the fauces were thickened and of the same general hue. The posterior pharyngeal wall was yellowish, with the blood vessels prominent and very red. There was atrophy of the glandular tissues of pharyngeal vault, and also at the base of the tongue. The arytenoids were enlarged but had a fatty yellowish appearance, instead of the bluish edematous look they present in laryngeal phthisis. The interior of the larynx was full and flabby, the tissues intruding into the caliber, while partaking of the yellowish hue of the rest of the membrane of the throat. The false cords seemed especially thickened. The vocal bands were normal, as was also the epiglottis with the exception of the pallor. In October last, the patient suddenly developed edema about her eyes, and the characteristic appearances of myxœdema. At the same time loss of hair under the arms and on the head was commencing. I had not seen the patient for four months, and the difference in appearance was quite striking. There was no albumen found in the urine. Suspecting that it was an undoubted case of myxœdema, I commenced treatment by giving 5 drops of the extract of thyroid gland in glycerin (Gibier) morning and night after meals, increasing the dose to three times a day at the end of a week. Patient was living on a simple diet but not restricted. At the end of two weeks a marked change appeared to manifest itself in the general comfort of the patient, and the spirits commenced to improve, at the expiration of three weeks the tongue began to become more manageable and breathing was easier upon exertion. In six weeks the patient began to lose flesh rapidly, had to get smaller gloves and shoes and commenced to have clothing taken in. The dose meanwhile had been reduced to 5 drops twice a day, and two weeks afterward to 4 at the same interval. At the end of six months there had been a loss of twenty pounds in flesh. The speech is fluent. No disturbance of ideas. Tongue perfectly free, thickening disappeared from the folds of fauces. Uvula, pharynx and larynx healthy. Color of the mucous membrane changed from the yellow waxy look to normal, and there persists but slight injection of the blood vessels. The measurements of one of the extremities taken by Mr. Riker for an elastic stocking before and after taking the thyroid extract show the following differences, and may be of interest

Ankle, 9 $\frac{1}{4}$, 8 $\frac{1}{2}$ Calf, 16, 14 $\frac{1}{2}$ Knee, 15 $\frac{1}{4}$, 14 $\frac{1}{4}$ Thigh, 21 $\frac{1}{4}$, 19 In the corset measure the hips appear to have decreased five inches, the waist three, and the bust four inches.

The above case is interesting in that it presented itself to a laryngologist. The difficulty with enunciation antedated the loss of hair and brittle finger nails by a year. Absence of albumen in the urine, no previous loss of hair, and no facial edema accounted for a late diagnosis of the true condition. However, to day the patient is restored to apparent health, looks younger by ten years, is as bright and cheerful as possible, and thoroughly enjoying life.

NOTE.—Patient is taking 3 drops of thyroid extract twice daily, and is in excellent health, Sept. 22, 1894.

THEORIES AND FACTS CONCERNING DEFLECTION OF THE NASAL SEPTUM

Read in the Section on Laryngology and Otology, at the Forty-fifth Annual Meeting of the American Medical Association, held at San Francisco June 5-8, 1894.

BY W. A. MARTIN, M.D.
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That the major portion of nasal troubles is caused by the deviation of the septum, I think will be admitted by those who have devoted much time to the treatment of nasal troubles and have investigated the causes of these troubles. As a nation the Americans may be said to be universally afflicted with deflected septa, and to this cause alone we may safely attribute 75 per cent of the nasal affections that are so frequent among them. I have been unable to find any extensive statistics concerning this trouble in the United States, but from my own observation I will venture to place the percentage of deviated septa as high as 90 per cent.

The only reliable statistics of any extent furnished on this subject are those of Mackenzie among the English and of Zuckerkandl among the Austrians. The first taken from the skulls in the museum of the College of Physicians and Surgeons in London and the latter principally from the cadaver. Other statistics have been published by Semeleder, Theile, Allen, Potiquet and others, but all in smaller numbers than the first mentioned. In this country, statistics so far as I have been able to find, have been limited to those who have applied for the treatment of some nasal trouble and consequently are of no value in determining the frequency of its occurrence, as there are untold numbers to whom it causes no inconvenience and a large proportion of the population who suffer in silence and never consult the specialist.

The statistics of Mackenzie show that in 2,152 skulls examined, 76.9 per cent of the septa were more or less deflected. Those of Theile, Semeleder and others taken in Germany differ only 1 or 2 per cent from Mackenzie. Statistics of Zuckerkandl show that in 370 skulls examined in Vienna, 53.2 per cent of the septa were deflected.

The most diverse theories have been evolved to account for this frequent condition of the septum, some of them most amusing when one considers the source from which they emanate. A few of the many I will quote are

"Blowing the nose habitually with the same hand.
Habit of sleeping with the same side of the face to the pillow.
Habit of putting finger in one side of the

nose Action of astringents, rickets, syphilis These causes I simply mention and do not consider it necessary to give them any further thought as causal factors in producing deflections of the septum The following causes are the ones accepted by the leading specialists of to day Traumatism, over-development of the septum, obstruction in one or both nostrils, heredity, evolutionary and devolutionary changes The first mentioned of these causes, viz Traumatism is the one accepted by Bosworth, who seeks to defend it in his text-book, "Diseases of the Nose and Throat," as being the most frequent source of the trouble

It is remarkable how readily a patient will recall some cause for the deformity, when informed that such a condition exists, in the shape of a blow on the nose fifteen years ago, a tumble down stairs, a frequent cause given by mothers is a blow from the baby's head, and numerous other causes are assigned too numerous to mention, but with which you are all familiar It is peculiar how sensitive individuals are concerning any deformity which is congenital, or for the source of which they have no natural or physical cause to offer They seem to consider it a blight cast on their lives by nature, but if they can offer a cause in the shape of some physical force, the defect is regarded as of little consequence or even as an insignia of glory, showing the prowess of the individual or the martyrdom through which he has passed Now deformities of the nose are no exception to this rule There is no feature about which people are so sensitive as the nose, reference to the shape will give offense, and many individuals who are perfectly satisfied with the external appearance of that organ will doubt the ability of the specialist, who informs him that the interior is twisted and contorted He immediately recalls some manufactured or almost forgotten reason for the existing condition, but the patient is willing to retract somewhat after being informed that his nose does not differ materially from the general average

Bosworth informs us that "an injury to the nose need not necessarily give rise to the immediate development of a notable deformity as in fracture, but it may set up a low grade of morbid action which going on through a number of years will develop a condition by which the normal function of the nose is seriously hampered"

Why this low grade of inflammation should continue for years, without causing any inconvenience excepting the resultant deformity and having caused the deformity should cease is not apparent One might give it some little consideration if we had only nasal spines and crista to deal with, but we know these are dependent on a curvature of the septum, and that condition would have to be first accounted for and could not be accounted for in this way The frequency with which we meet with this deformity is the strongest argument against the theory of trauma Certainly 90 per cent of Americans and 75 per cent of the European population have not suffered sufficient injury to their noses to produce this almost universal defect in this particular portion of their anatomy

In a neat little brochure, Mr Mayo-Collier distributed broadcast throughout the land his idea of the real cause of this deformity I quote here the introductory notice "The ready and full acceptance of my views on the causation of deflections of the nasal septum by the very competent tribunal to

which I submitted them has lent some warrant for my placing this small monograph before the profession"

The tribunal referred to is the British Laryngological and Rhinological Society Certainly, if such a competent tribunal has passed favorable judgment on Mr Mayo Collier's views I can not ignore them

The sum and substance of Mr Collier's conclusions is, that the deflection of the septum is caused by a stenosis of one or both nostrils and the resultant atmospheric pressure acting on the unoccluded side, thus pressing the septum toward the opposite side The theory is pretty, but it does not work in practice In the first place most specialists agree that the septum is seldom if ever deflected in the posterior third, and that the curvature is almost exclusively confined to the anterior two-thirds If the deflection is the result of atmospheric pressure, the posterior third would certainly be more exposed to such pressure than any other portion of the septum In every case of stenosis, where we find a curvature in the middle or anterior third, the posterior third would be exposed to the same or greater pressure than the part affected

One of the favored points of deflection is in the cartilage Jurasz asserts it is always there, now certainly a stenosis never occurs anteriorly to the cartilage, it may occur as far forward as the cartilage, but then this would not furnish the necessary condition for a deviation that is confined mostly to that portion

Artificial stenosis, as in the case of foreign bodies retained in the nose, furnish conditions most favorable for the production of a deflection if Mr Collier's views are correct Since reading his monograph I have had five such cases under treatment where the foreign bodies had been *in situ*, periods ranging from three months to five years, in each and all of these cases, the deflection was toward the unoccluded side The cases were all children of from 3 to 9 years of age, a time of life when the septum is most pliable and when it would be most easily affected by such pressure

Mr Collier lightly disposes of polypi in "one breath" Certainly they deserve some consideration as one of the causes of stenosis At the time of writing I have under my treatment a boy 9 years of age, who has had the right nostril obstructed for a year or more The entire right side of the face is undeveloped The septum, however, is very little if at all deviated

Because 80 per cent of savages and aborigines have symmetrically placed septa, Mr Collier reasons that civilized people are more prone to colds and injuries, etc, that produce stenosis, which leads ultimately to the deflection of the septum A great many scientists hastily conclude, that because civilized people are subject to affections from which the uncivilized are free, that the manner of living must necessarily be the cause Civilization and refinement I admit are accountable for some of the minor ills from which mankind suffer, but there are great laws that control the human race and which are unaffected by the manner of living, and certainly there is no law that produces a deformity that has such serious consequences as the deformity under consideration, that does not have some more reasonable explanation that it is simply a result of civilization

Certainly, we do not live under any more unfavor

able conditions than the Chinese, and again take the statistics of the American negro as stated by Allen. Here is a race living in the same country and climate and under more unfavorable conditions than the majority of Americans, and still the statistics show that only about 20 per cent are affected with deviation of the septum.

As a "liqueur" to the feast, Mr Collier quotes the results of Ziem's experiments on animals as proof of the efficacy of the great cause for which he contends. No one attempts to dispute the effect of stenosis of long standing, but unfortunately Mr Collier has mistaken the cause as an effect. The most frequent and most permanent form of nasal stenosis is the deviation of the nasal septum and it is secondary to no other form of stenosis, it is the form that produces asthenopia, and assymetry of the face.

Over-development of the septum as first mentioned by Morgagni and later by Chassaignac, and accepted as a satisfactory explanation of the cause of this deformity by numerous specialists of to day is decidedly unscientific. If there is a tendency to over-growth of the nasal septum we must have an explanation of the cause of this over-growth.

We are willing to admit that in the case of a deflected septum there is a profligacy of Nature in supplying such an abundance of material, but it is the wherefore we are seeking and not the fact that it is there, which is palpable to every observer.

Heredity as a cause is equally unscientific. It is no cause, it is merely a law that perpetuates the deformity. I can relate a circumstance where three brothers and one sister were all affected with exactly the same form of deviation. The external appearance of the noses was so much alike that one could call any of them by name, having been introduced to one of the four. It was a family nose inherited from their father. Heredity explains it thus far, are we to be satisfied with the explanation, or shall we seek farther and find that it descended from one generation to the other until the original of this type of the family nose is found?

Evolutionary or devolutionary changes. Here is the field in which our researches are most likely to be rewarded. There have been several searchers in this field working mainly in the same direction. Among the more prominent are Potiquet, Bendelack Hewetsen and Zuckerkandl. They commenced with the same theory, namely That the condition was the result of a change from prognathism to orthognathism. Zuckerkandl merely suggests it without offering any particular reasons.

The paper read by Bendelack Hewetsen a few months later than the monograph read by Mr Collier, shows that there was at least one member of the British Laryngological and Rhinological Society who did not accept the latter gentleman's theory as absolutely conclusive. Hewetsen in his paper discussed "The Relations between the Various Forms of Nasal Stenosis and Deafness." He found that nasal stenosis was associated with a narrowness of the jaw, crowding of the teeth and an elevation of the palate. This narrowing of the modern face, "the ultra refined face", as he terms it, he attributes to the fact that civilized men partook habitually of a much softer food than that which fell to the lot of savage man. He had examined large numbers of natives of the Zanzibar coast of Hindoos and lastly large numbers of Arabs in Morocco. In none of these people did he find any nasal

stenosis. It is to be presumed that there being no stenosis in these people examined, that the septum was quite or nearly symmetrically placed. Mr Lenox Brown very correctly observed in the discussion of the paper that "the diet of lentils and curries which Hewetsen said formed the principal food of the people examined, did not seem as difficult of mastication as the average specimen of meat, which the modern European was called upon to masticate. The Mongolians certainly subsist on softer food than the Europeans.

Dr Potiquet gave the result of his researches on this subject to the Paris Society of Laryngology, Otology and Rhinology in July, 1892. His researches were conducted on the 400 skulls in the museum of the amphitheater of the hospitals. I quote from a report in the *Journal of Laryngology* "His conclusions are that in the Parisian, the osseous nasal septum tends to incurve so much the more, as the movement of rotation of the pre-maxillary around the point of junction of the posterior extremity of the vomer with the body of the sphenoid taken as a center, is more marked. This conclusion may be regarded as a verification applied to a very limited group, of a law common to the whole series of primates, to wit That the nasal septum has so much the greater chance of lateral incurvement as the axis of the face is itself the more inflected in its antero-posterior direction. As this axis is more and more inflected as one rises from the anthropoid to the European male, the septum tends to incurve laterally more and more as one follows this ascending series. Lateral curvature of the septum therefore appears to be an evolutionary phenomenon related to the degree of antero-posterior inflection of the axis of the face." Farther along in the same article the writer remarks "It would not be extravagant to say that a man has only exceptionally the septum which belongs to him."

It is difficult to argue this point without fuller statistics at one's disposal. For my own part, however, I do not believe that the rotation of the axis of the face in the European male is greater than in the case of the Hindoo and Arab, who Hewetsen claims are seldom if ever troubled with stenosis, but Potiquet says "It is a law applied to a very limited group," and I judge that in this way he excludes all except the Europeans and their immediate descendants, but Nature's laws are not elective. If this condition is the result of evolutionary changes pure and simple, a change produced simply by the progressive or retrogressive state of man to accommodate him to a new condition of things, then it is the only instance I have been able to find where there has been such a marked assymetry of development as to impair the usefulness of the organ. We have instances where parts have atrophied when they were of no more use or left in disuse, but as far as I am aware none where there has been a hypertrophy.

It is possible that the nose, as an organ of sense, has not the magnified function that it had when man was in a less civilized state, but the peculiar shapes that characterize different races are probably not so much a result of the increased or impaired functions of that organ as a result of sexual selection. The nose, one might say, is the center of attractiveness in the determination of beauty in the eyes of most races. This being the case it is not strange that in the development of the different races we find in

their perfect development a type of nose that varies very little in the different individuals of the same race

There is no feature or characteristic of a race that is so typical as the nose. The features of the purer races as the Mongolian, African, or Polynesian are so much alike that it is with the greatest difficulty that even the most skilled physiognomists are enabled to distinguish between individuals without a close acquaintance with them. This fact is aptly demonstrated by the difficulty we have had in finding some positive way of excluding our Mongolian brother from the confines of our country.

From the statistics at our disposal of Mackenzie, Zuckerkandl, Adams, Hewetsen and others, we find that the nasal septum of these different races, namely, Chinese, Japanese, Malay, Hindoo, Arab, American Indian and Polynesian varies as little in shape as does the external appearance of the nose.

Among the Europeans and their immediate descendants we find that there is no characteristic type of nose. In Paris, in the classification they have adopted for the recognition of criminals, they distinguish nine distinct types of noses and with subdivisions have as many as twenty-seven. These distinctions are from—the outline of the nose, convex, rectilinear, or concave—base of nose, ascending, straight or descending, these in the different combinations make nine varieties and either of the combinations may be either thin, medium or broad. The interior probably offers greater variety than the exterior in so far as the septum is concerned. There are distinct types of noses, however, preserved in limited areas of Europe that were originally typical of a grand subdivision of the Caucasian race, as for instance the Roman, the Grecian, the Semitic, and again the other subdivisions as the Teutonic, Slavonic and other tribes that at one time had distinctive noses. The same law that produced the Mongolian or African type was effective in shaping the nose of the Roman, the Greek or the Teuton. It was the outcome of centuries of exclusiveness, and I venture to make the assertion that these people were no more troubled with the deflection of septum and the resulting stenosis than the Mongolian and other purer races of to-day. Their noses were the outcome of natural evolutionary changes.

How about the European population of to-day? The modern European and the immediate descendant to a greater extent represents a composite anatomy. The work of centuries in building up and fashioning tribes of distinctive types of features has been undone in a comparatively short period by the mingling and mixture of these distinct tribes. The commingling has not been limited to the people of the Caucasian races, but other races of a distinctive type, as the Mongolian and African, the American aborigines and others equally as distinct, have all united in lending characteristics to the modern European and American. I need not enter into the genesis of the European. It is a matter of history and accessible to all. The mixture, the result of conquest and slavery in ancient times and in later years of emigration and intermarriage between persons of different nationalities has equalized to a greater or less extent the distinctive type of features.

If we could examine the skulls of the purer ancient types as the Roman, the Grecian or the Semitic, I dare say we should find the deflected septum as rare

among them as among the purer races of to-day. If we should take the septum from the skull of one of these people and attempt to fit it into a Chinese or negro skull we would assuredly find a misfit, but this is the condition imposed on Nature by the mixture of races and a condition that is not likely to be modified for centuries to come as the evolutionary changes necessary for Nature to accommodate herself to this innovation will be slow, as all evolutionary changes are.

We might accept it as a law that the straight or symmetrical septum is distinctive of a pure race, while the deviated septum is characteristic of a mixed race.

Such being the case, where would we expect to meet with this deformity most frequently? I think that you will all agree that the people of the United States are the greatest sufferers in this respect. In England, Mackenzie's statistics show that 76.9 per cent of the population suffer with this deformity. If we could have statistics from Russia where there has been less of a mixture of races than in other European countries we would probably find the straight septum more the rule. Zuckerkandl's statistics show only 53.9 per cent, as we would naturally expect, as the farther east we travel the nearer we approach to original racial types. In the purer races as the negro and the Mongolian, we find the same shape to the nose in the child that we find in the adult, and other things equal we see no variation from the original type. The child resembles both its father and its mother, and in fact the whole race as far as features are concerned. In the European and American, however, the case is quite different, here there is apt to be a reversion to ancestral type. The offspring may resemble any progenitor as far back as its simian ancestors and excite no particular comment. An American with a Roman nose who takes for a spouse a woman with a *retroussé* nose may find in his several children as many different shaped noses. The only fact he can safely predict is that one and all will have a deflected septum. In a well regulated Roman, Grecian or Semitic family I venture to remark that if any of the children had had a *retroussé* nose that there would have been an investigation set afoot.

That deflected septa are as frequent as 20 per cent in non-European and non-American people is readily accounted for by the fact that even in the most isolated races there is a certain percentage of mixture. There are few people whom we have the opportunity of examining among whom the whites have not mixed. Missionaries, adventurers, travelers and shipwrecked persons have gained a footing in even the remotest corners of the earth and their imprint is found stamped in the nasal septa of the inhabitants.

Another important factor must not be overlooked as accounting for a certain percentage of the deformed septa both in the civilized and uncivilized races, that is owing also to a disparity in the development of the cranial and facial bones. We find not infrequently that the cranium is small in proportion to the development of the facial bones, owing to the too early ossification of the cranial sutures. This need not be sufficient to bring the individual within the limits of cretinism, but sufficient to be an important factor in causing a deformity of the septum. The most deformed septa I have ever seen were in

individuals belonging to this class. Three cases I recall where the individuals escaped only with their reason. Two had the peculiar facial expression of those affected with pharyngeal adenoids, but they were in no way improved either as to facial expression or as to mental condition by the removal of septal spines, crests and enlarged turbinates which furnished free breathing room through the nostrils, thus proving rather conclusively to my mind that the disparity in development in these cases was the cause of the nasal stenosis, and that the latter condition had very little effect on the mental condition, as one might infer on first thought.

The deviation of the septum resulting from anomalies in development occurs during the time of the second dentition from the seventh to the fourteenth year, it may become more pronounced from then up to the eighteenth or twentieth year when ossification in the skull is completed. It rarely if ever occurs before the fifth and never after the twentieth year, unless from pressure or traumatic causes. It occurs during the years of greatest development of the brain, and consequently with the greatest expansion of the cranial and facial bones. Whether during this period the space between the ethmoid and the superior maxillary is abridged, or whether there is a greater development of the septum than the space admits, or whether both these factors come into play, I am not prepared to state. Up to the time of the second dentition there is a similarity in the facial appearance of European and American children, but at this time there commences a variance in the nasal shapes that does not occur in the children of unmixed races. The mixed races seem to be affected by other anomalies in development of the facial bones that occur principally during the same period, namely, the narrow and shortened jaws and the high arched palate, the latter more apparent than real, as the nearer approach of the alveolar processes makes the palatal arch seemingly higher. These anomalies all seem to be more clearly explained by the blending of different racial types than by evolutionary or devolutionary changes from a prognathous to an orthognathous type. The whole matter is one of theory, but the weight of the evidence is in favor of the former, because we know it has been and is occurring continually while the latter supposition is purely theoretical.

FIBRO-LIPOMA OF THE PHARYNX

Read in the Section on Laryngology and Otology at the Forty-fifth Annual Meeting of the American Medical Association held at San Francisco June 5-6 1894

BY JOHN O. ROE, M.D.

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REPORT OF CASE

Benign tumors of the oro-pharynx, involving the posterior wall alone, are of comparatively infrequent occurrence. Judging from the cases reported, and from my own experience, lipomatous tumors located in this region are the rarest of all. The following exceedingly interesting case, that of a large fibro-lipoma of the pharynx came under my observation last January, and is quite worthy of record.

Mrs. R., 49 years of age began nine months before I saw her to experience a sensation of roughness and fullness in her throat, attended with difficulty in swallowing, and associated with pain extending to both ears. While remaining quiet she experienced no special discomfort in her throat

but on attempting to swallow, the sensation of constriction about the throat and the pain in her ears would be frequently quite severe. This difficulty in swallowing began with the taking of solids, particularly in large pieces, and gradually increased until the time she came under my observation, when she could swallow liquids only and these only in small quantities. Owing to this great difficulty in swallowing she had become emaciated, and was rapidly losing flesh and strength. An examination of her throat revealed a large swelling projecting forward from the posterior pharyngeal wall, nearly filling the pharynx. It extended vertically from a point opposite the base of the uvula to a point opposite the arytenoid cartilages, and laterally from one posterior pillar to the other. This enlargement was somewhat ovoid in shape, the larger portion being above, and covered with mucous membrane, indicating the existence of a growth located behind the pharyngeal wall. The mucous membrane covering the swelling was somewhat pale from the pressure of the growth beneath, but otherwise presenting no unusual appearance, and there was no evidence of previous ulceration or disease of any nature.

On palpation, this swelling had a soft doughy feeling, similar to that of an abscess, but the history and appearance gave no suspicion that this swelling was of that character. There was no history of a specific disease, and the patient had met with no injury to the pharynx of which she was aware. Thinking, perhaps, the swelling in her throat might be a collection of enlarged post-pharyngeal lymphatics, due to some unknown specific trouble, I placed her on vigorous specific treatment for a time. This treatment, however, had no effect upon the swelling in her throat, therefore, without doubt, the swelling was not of a specific nature. The growth to all appearance was a lipoma, and I accordingly advised its removal, which was done the following day. Local anesthesia with cocaine injected and applied to the surface was quite sufficient to relieve the pain. An incision about an inch and a half long was made along the median line down to the tumor, which lay encysted just beneath the submucous connective tissue. I then endeavored with a thin curved elevator to separate the tissues from the growth and thereby to enucleate it. This I succeeded but partly in doing and, owing to the fact that the thin portion of the anterior wall of the growth became ruptured, a portion of the contents escaped. This was preserved for further examination and found to be composed of fat. In conjunction with this substance other tissue of a firm character was found, which was afterward shown on examination to be fibrous connective tissue.

It was with some difficulty that the growth was separated from the surrounding connective tissue, and along the posterior surface it was attached and firmly adherent to the pre-vertebral fibrous tissue. The attachment of the growth extended throughout the posterior surface from a point opposite the base of the uvula to a point opposite the arytenoid cartilages, being the area that the growth occupied externally as indicated during the first examination. It was with considerable difficulty that this attachment was removed from the vertebrae, requiring careful dissection. The removal of the growth was attended with very little hemorrhage, no vessels requiring ligation, and capillary oozing soon subsided.

The whole cavity after being thoroughly cleansed with a solution of bichlorid of mercury (1 to 5000) was completely packed with iodoform gauze, not only to render the parts thoroughly aseptic, but also to prevent food from entering the sac. Notwithstanding the fact that the sac was as completely filled with the iodoform gauze as it had previously been with the growth, the patient could swallow much more easily than before the operation. She was placed upon sterilized milk diet which was maintained until the parts had healed.

The cavity left by the removal of the growth was allowed to fill with granulation, requiring about two weeks. At one spot there was a portion of the attachment of the growth to the posterior wall that had not been completely removed, this was destroyed by the galvano-cautery. At the end of three weeks the surface was completely healed, leaving only a slight scar along the median line, and since that time there has been no evidence of any return of the growth.

This growth was evidently a combination of a lipoma molle and a fibro-lipoma, in which the fatty portion had become liquified by the heat of the body and by the motion and pressure to which it was subjected. A case similar to this one, in nearly all par-

ticulars, is reported by Taylor¹ In Taylor's case, the tumor was mistaken for a post-pharyngeal abscess and incised. It passed down so far behind and projected so far on each side of the larynx as to cause dysphagia and dyspnea from pressure on the larynx. Tracheotomy gave no relief. In the case I have described the patient suffered from dyspnea, although not from pressure upon the larynx but from partial obstruction of the larynx, and also of the pharynx by the growth interfering with the ingress of air.

DIPHTHERITIC CRICOID PERICHONDRITIS AND NECROSIS

Read in the Section on Laryngology and Otology, at the Forty fifth Annual Meeting of the American Medical Association, held at San Francisco, June 5-8 1891

BY PROFESSOR RAMON DE LA SOTA Y LASTRA, M D
SEVILLE

The idiopathic inflammation of the laryngeal cartilages is considered a rare affection by the greater part of the authors, who instead affirm that the laryngeal perichondritis and chondritis, as manifestations of certain constitutional illnesses, pocken, typhoid fever, syphilis, lupus, cancer, tuberculosis and mercurial intoxication, are sicknesses which are observed frequently. I think that the idiopathic perichondritis appears many or more times than the symptomatic, and if we do not take notice of it with more attention, it is because it terminates generally by resolution, and passes without being noticed, and we believe it to be an inflammation limited to the mucous membrane, and to the immediately sub-mucous tissue, which penetrates into the same cartilage. For which it is called intense inflammation of the arytenoid mucous membrane, that which not only invades the immediately submucous tissue, but that which reaches the crico-arytenoid articulation, beginning in many cases the phlogosis by the same articular surfaces after that it goes on to attack the mucous membrane, this being the consecutive inflammation, and the arthritis the primitive one.

Among the causes of the consecutive perichondritis only two authors notice the diphtheria, Gottstein and Bosworth, the first saying, speaking from the etiology of the perichondritis, that this "can appear as joined to an ulcerative process already existent of a diphtheritic character, but without stating if his affirmation is purely rational or deduced from the experience, and the second cites the diphtheria as a cause of the inflammation of the laryngeal cartilages, because one of the 33 cases of perichondritis observed by him was produced by the diphtheria. Volume II of the *Journal of Laryngology*, pages 366 and 367 contains an extract of a case published by Jacoborvitsch, from St Petersburg, in the *Archives für Kinderheilk*, Vol 10, No 1 "It was a child a year and three months old, which suffered the scarlatine. The diphtheria of the throat appeared several weeks after, and the diphtheritic membranes persisted on the tonsils and upon the pharyngeal vault during two months. There was also increasing stenotic respiration. Tracheotomy was performed, and the child died. In the necropsy was discovered perichondritis of the cricoid. The pharyngeal mucous membrane was covered with pus and membranes."

The *Internationales Centralblatt für Laryngologie*, published at Berlin from 1889 till 1890, page 189, copies from the *British Medical Journal*, Sept 22, 1888, a case of the necrotic perichondritis observed in a pig by Proctor S Hutchinson, of London. "The animal suffered from symptoms, which resembled those of the croup. It was not founded on motives to diagnose the acute illness. The animal died, and necropsy was performed. It was found with an inflammation, which was extended around the cricoid cartilage with partial necrosis of the same. A very great part of this cartilage fell down, and only remained a little part of the lower and anterior portion. The suppuration was insignificant. The necrosed piece was buried in a tissue of soft granulations. At last existed a general edema of the larynx, which explained the dyspnea."

This is all that I could find in the medical literature, which says anything with regard to the appearance of laryngeal perichondritis produced by the diphtheria, not even Knight, (who, in the eighth annual meeting of the American Laryngological Association, held at Philadelphia in May, 1886, with motive of a case of perichondritis, studied the causes of this affection in a splendid work) mentions diphtheria.

I have recently had occasion of observing the fatal consequences of this complication of diphtheria (which I think is not very rare) and by it have been many deaths attributed to other different causes. But before making more reflections, I will refer to the history of an interesting case for more than one reason.

February 4, 1894, I was called in consultation by the well-known physician, Reyero, on a child of 23 months, whom he found with all the symptoms of diphtheritic croup, and he recommended me to take the necessary instruments in case of necessity of performing the intubation, as he supposed.

At 8 o'clock in the evening we assembled at the house of the little invalid, who six or eight days before had shown symptoms of the bronchial catarrh, according to the statement of the parents, who had not been alarmed and had not called in the physician until that same afternoon, because they did not think it was fever, and notwithstanding that he did not like to eat, because he felt a pain in swallowing coughed frequently, was hoarse and was unable to get up, but breathed well, they had confined themselves to have him protected from the cold, poulticed in the neck, and giving him some warm milk. They gave him also two emetics of ipecacuanha, one every second day the latter one that same day, and notwithstanding the two operated well, as the illness not only remained, but increased until the difficulty of breathing inspired the parents with some dread, they resolved to call the physician.

The state in which Dr Reyero found the little invalid five hours before the consultation, was a little more or less the same in which we found him when we joined for the consultation, and believing the child to be in a very grave condition, Dr Reyero had the consultation immediately. I noticed that it was a larger child than its age seemed to warrant, and seemingly vigorous. He slept in his mother's arms, with difficulty breathing noisily in the inspiration and in the expiration, the forehead was covered with a cold and viscous sweat, a livid line existed around the eyes and the mouth, the cheeks were very high

¹ Lancet, London, 1876, Vol II, p 685

colored, but the ears, the lower part of the face and the nose were pale, the wings of these latter moved when he breathed, the supra and infra-clavicular, and supra-external fossæ, and the epigastrium fell down profoundly during the inspiration, the scapular, sterno-cleido-mastoides, pectoral and abdominal muscles concurred with their contractions to introduce the greatest possible quantity of air in the lungs. The respiration was shallow and hasty (38 every minute), the pulse was feeble and quick (112 every minute), the skin of the extremities was cold, the nails were blue, and the axillar temperature arrived to 38.9 degrees. Examining the mouth, appeared a white and large false membrane, which covered the soft palate, the uvula, the two pillars and the two tonsils. The rest of the mucous membrane of the palate and the pharynx was very red and friable, and to the least light friction bled. From the excoriated nose flowed a bloody ichor, but the false membranes were not seen there. At auscultation we noted a noise of large bubbles as much in the anterior part as in the posterior part of the chest. Our diagnosis was pharyngeal and laryngeal diphtheria in the asphyxia period, and complicated with intense and extensive bronchitis.

We made a very dangerous prognostic, not only by the advanced and extensive suffering, but because the grippe was existing in Seville at that time. We feared that the bronchial catarrh would assume an infective character, if by chance it had not yet assumed it. Notwithstanding, we agreed that it was necessary to perform the intubation immediately, in order to free the child from the imminent asphyxia, to separate the false membrane with a ball of very firm sterilized cotton, to irrigate the throat and the nose with a solution of carbolic acid (1 to 100), to touch the sores of the diphtheritic plaques with another ball of sterilized cotton moistened in a solution of the absolute phenol in the sulpho-vicinat of soda (20 to 100), these three latter were to be done every hour. The following mixture

R Hydrargyri bichlorid 01 gram
Tinct ferri chlorid 8 cc,
Potassi chloratis, 4 gram
Glycer, 60 cc.,
Aqua, 120 cc.,

was given, a teaspoonful every hour, and we advised that nourishment should be often taken, taking care to place the invalid with his head downwards, whenever he took any fluid. Moreover the bed was surrounded with sheets, making like a tent, and burning within the sheet two grams of calomelanos every four hours.

All was performed as we ordered. The intubation was made very easily and promptly, putting the tube, No 2, of the scale of O'Dwyer, corresponding to the two years which the child had. But at the first fit of coughing after having placed the tube this was expelled with force and to a great distance, which showed me the necessity of placing another and larger tube, and without difficulty I put that of the No 3, and the little invalid remained breathing, coughing and discharging mucosities by the tube with great facility, and without showing any grievous symptoms.

The first night he was very agitated, the tracheal rale was heard outside the room, and the temperature was raised to 39.8 degrees. The succeeding days at 10 o'clock in the morning the fever went down suffi-

ciently, for it descended to 38 degrees, but it rose again during the night, the child did not wish to eat, it was sorrowful and did not speak. Notwithstanding, the respiration was performed easily, and the false membranes were every time reproduced with less vigor.

On the sixth day I determined to take the tube away, that I had hesitated to take away before, watching the bulk of the introduced instrument. The child passed the day better than the anterior days, he had sat down at table with his parents and had wished to eat what he saw, though he suffered much in swallowing it, the diphtheritic sores of the throat had totally disappeared, but the respiration (a condition that should not be forgotten, by what happened after) was noisy, notwithstanding that it was performed easily as the tracheal rattle and the axillar temperature was raised to 38.7 degrees. The extraction of the tube was performed quickly and well, but in the moment that it was taken away from the larynx, and when I believed that by its friction against the posterior laryngeal wall, the violent fits of cough commenced which expelled the materials lodged in the trachea, I observed with great disgust that they were carried down by the current of inspired air, and afterward the child began to suffer the peculiar paroxysm, precursor to the asphyxia. I expected that this dyspnea would disappear by degrees, as I have had occasion to observe it in other operated persons, and examined the tube, which was completely obstructed, and saw a piece of the macerated false membrane formed a cap or a capsule in the inferior extremity of the instrument. I introduced the obturator of the tube and there came out a dense mass in shape of a cylinder a veritable mold of the lumen of the tube, composed of detritus of false membrane, by semi-coagulated mucus and probably by some food. I called my learned colleague's attention to all, and he asked me from what time this material had filled the tube. I could not tell him, by the consistency of the plug, which did not break when it fell to the floor, I ventured to say that the tube had been obstructed from twenty-four to forty-eight hours.

When I was occupied in these examinations the state of the child grew rapidly worse. I observed it by the noise which the air made in passing the glottis, and my friend Reyero advised me of it, at first with tranquillity and the second time with urgency, because the little fellow was suffocating. I was already trying to place a thread in the tube in order to repeat the intubation, but the moisture of the instrument and of my hands made it very difficult to slip the delicate thread of silk in the small hole of the tube. I could not do it. Reyero was alarmed, and with reason, because the face of the child was violet, and he told me again of the danger, and for that reason I left the silk, because there was not a moment to lose, and, placing my confidence in God, without silk and without gag, I performed the intubation, doing it with great facility and at the first attempt, and beginning immediately the artificial respiration, because the child could not get breath and all its skin was covered with a cold and viscous perspiration, and the muscles completely relaxed. We obtained in three or four minutes of the artificial respiration the natural one well restored. The child slept after the danger which he had run, and we were smiling after having been in such great anxiety.

We attributed what happened to the aggregation upon the wing of the trachea of layers of the false membranes and of a great quantity of semi-concrete mucus, and we decided to leave the tube for two days more, holding same medication, but stopping the painting with the solution of phenol, because the diphtheritic sores had disappeared.

Thirty eight hours were passed without any accident, the temperature had been normal since the following day, the same in the morning and evening. The child wished to eat, but the deglutition was always painful, the respiration was accomplished without noise and suddenly at a fit of coughing the tube was expelled. I was called and went with the haste which was natural after what happened two days before, and when I arrived at the house of the invalid, which is very far from mine, I had the consolation of finding the child respiring, though with great pain. I was surprised that the tube was expelled so soon, because it was much larger than usually used in a child of that age. The dyspnea reappeared the moment the tube was extracted, and increased until nearly asphyxiated, although the lumen of the instrument was clean and we could not hear the tracheal rattle but we could hear the whistle which the air made in the glottis, as much in the inspiration as in the expiration. I performed intubation for the third or fourth time, and all remained as if nothing had happened, but I was very displeased, because I thought that it was caused by a post-diphtheritic paralysis of the posterior crico-arytenoid muscles, and so I told Dr. Reyero, who coincided with me in the diagnosis and also in the prognosis, we then left the house sorrowfully thinking of the paralysis in such little muscles where perhaps it had never been before. Knowing how rebellious and long are these fits of paralysis, we did not dare to say how long we should have to leave the tube in the larynx, and the inconveniences that were present by the paralysis of the dilating muscles of the glottis. Notwithstanding, as that day the dyspnea had not been as early and as severe as two days before, I hoped that the tube inciting the part would determine the contraction of the posterior muscles. In thinking of these things we arranged for a new consultation in two days, in order to take away the tube, besides he did not expel it as before, and resolving to carry one of the shortest tubes and of a great diameter to substitute for that placed, in case it were necessary.

On these two days nothing happened worthy of mention, and to the disgust of the parents I took away the tube, and the scene of the latter day was repeated. I then placed the third tube of the short ones, and left the child all right.

Two more days were passed with tranquillity, the only unpleasant phenomenon was the pain in the larynx sustained, in my opinion, by the long permanency of the tube in the larynx, and so great was my desire of taking away the instrument, that I extracted it in the hope of finishing my task that evening, but if there was a moment in which it seemed that the respiration was performed easily, that passed, and the peculiar respiratory whistle was repeated. With disgust I listened to it, but I did not wish to repeat the operation. Reyero looked at me and said "I do not wish the child to remain like this, I have very much to do and can not lose the time. I beg you to repeat the intubation." He was right, and I obeyed.

The child resisted all our maneuvers, thus as soon as he saw the gag he closed the teeth, and there was no human force that could separate his jaws, we vexed and annoyed him every time that the gag was applied. On this occasion the maneuver was something more tedious than the anterior ones, and a tooth was knocked out, the little invalid shook the head in the moment of nausea, so notwithstanding our efforts to subdue him I was only able to place the gag in an imperfect manner. So I performed, but when I put my finger upon the head of the tube in order to withdraw the obturator, I observed that the larynx fell down until I could not touch it with my finger. I stopped in suspense. What had happened? Where had the tube fallen down? That tube of which the diameter was triple, that which corresponded to the age of the child? To the subglottic part? To the trachea? Impossible, because the shortest tube placed by Brothers in the larynx of the dead body of a child could not be made to go down to the trachea, far less could this large tube fall down so small a larynx and with so light an effort. Guided by this reasoning I took away the thread. Notwithstanding so nervous a person, as I, can not rest under the power of the imagination without suffering dreadfully, and for that reason I decided to take away the tube. I introduced the extractor and putting it well down I managed to seize the tube and take it away. I had nothing to dread, but it was necessary to repeat the operation, and as the child had the mouth open, and recollecting what I had done a few days before in very exacting circumstances, I wished to repeat the operation, that is, I wished to introduce the tube without putting in it the thread of silk, but the tube, large and short, was separated from the obturator, and before I could seize it, the tube fell down the esophagus lengthwise, and penetrated into the stomach. A similar contrariety bothered me very much, though I knew already that the tube would sooner or later be expelled by the ano, and in the same instant I seized another tube of the largest and shortest, put in it the silk, and placed it in the larynx. The tube was larger than that which had been slipped by the esophagus, and, notwithstanding, I felt it slip under my finger, just as the other one had done when it fell down, but yet I did not trouble myself, because I knew that it was upon the vocal cords, notwithstanding I was not able to explain what happened, for the paralysis of the abductor muscles was not sufficient reason for this rare phenomenon. That happened February 23, and we agreed that the parents should carry the little invalid to my home on the 26th, in order to begin the application of electricity to the paralyzed muscles.

By misfortune, I woke on the 25th with a great superciliary neuralgia, which obliged me to remain in bed. On the evening of this day the child expelled the latter tube, so extraordinary large for its age, and the father called me in order to help his son, who was suffocating. As I could not rise from the bed, so I besought my dear disciple and expert laryngologist, Gallegos, to go and do what he believed necessary for the child.

My friend went instantly, found the child in asphyxia, and introduced the tube which had been expelled two hours before. In introducing it he observed what I had observed already, that is, that the tube under the pressure of the finger slipped by

the larynx to a place where it could not be touched by the finger. That surprised him as it had surprised me, but he had not time to pre-occupy himself, because he had scarcely taken away the thread than a fit of the cough expelled the tube out of the larynx. This scene was repeated six times, on the third, after the issue of the tube was a moment of imminent asphyxia, a whitish body appeared between the teeth in the middle of thick and sanguineous mucosities, which Gallegos seized, believing that it was a tooth, as the anterior, separated. When he had it in his hand he saw that it was not a tooth, but a white opaline substance, from two millimeters of thickness by ten and seven of length and nine of breadth, irregular, concave on one of its surfaces, convex on the other, with two even and round borders, one superior and the other inferior, and with two dentated extremities. He considered what it might be. At first he thought it was a piece of epiglottis, but when he examined this with the finger, on making a new intubation, he observed that it was intact. He thought then if it might be a substance thrown from the stomach, but none of the food that the child had taken could contain anything of that size and form. Believing that it was gristle he wrapped it carefully in a paper and continued attending to the patient. Tired and weary because the tube was always thrown out by the cough which the extraction of the thread caused, he decided to leave it, reminding the mother not to loosen the thread at all during the night, so that the child could not pull it with his hands and extract the tube.

On the following morning Gallegos went to see the patient and found that he could take the thread without the tube being expelled, and he came to tell me all that had passed and to give me the tube which had fallen into the stomach, and that before twenty-four hours it had been expelled by the ano. Also gave me the strange body taken between the teeth on the preceding night. I staid in bed suffering from neuralgia, heard the relation of my friend, seized that which he gave me, and looked at it carefully. It was already dry, and was transparent and hard, rolled upon one of its surfaces, and had all the appearance of a large psoriasis scale. "I do not know what it is," said I to him.

"Nor I either," he replied, "but I believe that it is a piece of gristle." "It does not appear to be so," I answered, "it resembles more a dried false membrane, or a piece of loosened and dried vocal cord." "You did not see it when it was fresh, then it was like a necrosed cartilage, and by its breadth I believed that it was the epiglottis, but then I have touched it with my finger, and observed that it was complete." "If it is cartilage, as you believe, it will be a piece of the cricoid gristle by its form, but leave it, for I can not speak. Please put it in my cabinet table." Observing the bad state in which I was Gallegos departed; and two hours after the parents of the child arrived at my consultation room with the little patient. He had expelled the tube, and I told them to take him to Gallegos, who performed the intubation, but three hours after another fit of coughing threw out the tube. The child remained without it all that afternoon and all the following night.

In the morning they called me, but as the neuralgia did not permit me to go, and knowing that the child was choking, that he could not swallow more than

fluids, and that his general state had grown much worse since I had last seen him, I advised them to perform tracheotomy, for it was seen that the paralysis of the vocal cords was so great that they wanted the necessary tension to retain the tube. I indicated this to the parents the day before, but they did not wish to have a bloody operation performed, yet at last, knowing that if the trachea was not cut their son would die, they asked of me the cannule of tracheotomy corresponding to the age of the child and with it they went to call Reyero. Unfortunately, he could not go to the child until 12 o'clock, half an hour before this, died, asphyxiated, that fine child who during fifteen days had passed through so many vicissitudes.

As soon as I was relieved from the neuralgia, I begged of my dear friend, the illustrious Dr Roquero, Professor of Histology in the School of Medicine of Seville, to make a microscopic examination of the body held by Gallegos. Roquero then immersed it in pure distilled water, and examined with the microscope a section, and saw that it was a hyaline cartilage, and that the piece would correspond to more than the posterior half of the cricoid cartilage. Effectually, after having soaked it in the water, the figure shown in the annexed photograph appeared clearly. The surfaces A and A correspond to the



articular facets upon which the arytenoid cartilages turn round, in the middle of the plate one can distinguish the projecting line that shows the union of the two halves of the seat of the ring, and in the sides you can see some rugosities indicative of the insertions of the posterior crico arytenoid muscles. This anatomic piece I conserve in a solution of hydrate of chloral, for it is equivalent to an necropsy, because it explains the phenomena observed during life, and puts out of doubt the existence of diphtheritic laryngeal perichondritis and necrosis.

If these affections are not quoted as frequently as they ought to be, without doubt we must impute that to people not thinking about more than the inflammation of the mucous membrane and the submucous tissue, as if further than that there is some insurmountable barrier to the simple inflammatory or specific process. As there is no such barrier, it will be permitted to suppose that in many cases the diphtheritic process advances to the perichondrium, and into the same cartilage, and if we do not observe its greater frequency the expulsion of the necrosed pieces, as in the present case, it is due to death happening before there was time for the cartilage to be carious in all its thickness, or that either a large or small sequestre could come out. Neither is it risky

to suppose that there have been cases of expulsion of the cartilaginous pieces, but unknown or not seen by the assistant persons

I have said that the piece taken from our patient is worth as much as an autopsy or more. It explains to me why the child was choked as soon as he expelled the tube. I imputed it the first time to the mucosities and detritus of the false membranes accumulated upon the spur of the trachea, the second time to a paralysis of the dilator muscles of the glottis of diphtheritic nature. This second time I was right about the paralysis, but I was wrong in its cause. The perichondritis must have appeared in those first eight days in which the child did not have the necessary medical attendance as proven by the intense dysphagia manifested by the little patient. In the moment of the first operation the cartilage must have been immersed in a bag of pus, formed under the perichondrium, the lateral parts of which were probably grown carious. During the first six days of the permanence of the tube, the perichondrium and cartilage stopped ulcerating and then the crico-arytenoid posterior muscles were lost in a great part of its extension. For this reason the vocal cords united as soon as the tube was taken away, and the air whistled in passing through, as much in the inspiration as in the expiration. For this reason the first tube, larger than was convenient for a child of that age was expelled in a fit of coughing, and the same happened with the second and third tube, and this fell down in the larynx, when it was pressed by the finger, because the larynx lacked resistance, the soft parts and the hard parts had lost the base of their support for it is well known that the cricoid cartilage is the fundamental cartilage of the larynx, and that all other parts of this organ rest upon it. When so considerable a part of the cricoid ring gives way as gave way in this case all the posterior laryngeal wall is put out of its place, the soft tissues are flattened or are united with those of the anterior part of same organ, the arytenoid cartilages necessarily sink and fall in the laryngeal vestibule, and the thyroid cartilage remains without control, through failure of its inferior adhesions.

But the case also obliges us to think that it is possible that one of the ordinary tubes of O'Dwyer can descend in the trachea, for Doctor Brothers, after his experiments, which prove the impossibility of the tube falling down by its own weight in the trachea, tells us that what principally impedes this descent is the enlargement or prominence of the cricoid cartilage in the anterior part of the ring. If that is so, then if a considerable part of the ring is separated, the retentive enlargement is wanting, and as the soft tissues are elastic, it is possible that the tube may penetrate to the tracheal conduit. If in this child this did not happen, it must be without doubt that when the sequestrum lost its relations with the anterior part of the ring, the diameter of the tube was too large. I, a decided partisan of the method of O'Dwyer, show this contrariety, which is neither little nor insignificant, because the fall of the tube in the trachea would oblige the operator to perform tracheotomy immediately in order to extract the strange body, which would place the physician who had already told the parents of the little patient that he knew a proceeding that would free the child from asphyxia without cutting, in a very delicate situation. Until now I never thought that I should

find myself in such a difficulty, for the future I shall have to dread it, although it may be rare, and I shall so tell the family.

But if this case has given me a motive for fear, at the same time it has given me another motive for confidence. All the authors, when writing about the serious accidents of intubation tell us about the obstruction of the tube by the false membranes or by other cause. In this child I found the tube completely obstructed when I took it away the first time, and notwithstanding the child breathed perfectly, and more, the dyspnea began as soon as I extracted the tube. How do you explain the phenomenon? I believe it very easy. Place two thin bands of caoutchouc of five millimeters in breadth by twenty in length in form of ribbons upon a tube, so that they are touched by its borders, but are not very tight. Between two bands is placed a little tube of O'Dwyer, and it will be seen that the ribbons remain separated two or three millimeters in front and behind the instrument. What more suffices for the respiration to be made with relative ease? A similar thing happens in the larynx. The vocal cords are relaxed as in the present case by the loss of resistance of its posterior insertions.

Finally, this history also teaches us that we must never try to extract a tube without having another prepared to introduce, if it were necessary, as happened the first time I took away the tube. If, the I have the misfortune, which I had in my latter intubation, that the tube gets out from the obturator and instead of falling into the larynx, it falls in the esophagus, the child would be asphyxiated with all certainty. I do not know if any one of those who have written upon intubation of the larynx have improved upon this precept, if none have previously indicated it, I now show it.

RETRO-PHARYNGEAL LYMPHADENITIS

Read in the Section on Laryngology and Otology, at the Forty-first Annual Meeting of the American Medical Association, held in San Francisco June 5-8 1894

BY W. E. CASSELBERRY, M. D.

PROFESSOR OF THERAPEUTICS AND OF LARYNGOLOGY AND RHINOLOGY, NORTHWESTERN UNIVERSITY MEDICAL SCHOOL (CHICAGO MEDICAL COLLEGE), LARYNGOLOGIST TO WESLEY HOSPITAL, ETC. CHICAGO, ILL.

It is now well established that retro-pharyngeal abscess arises ordinarily not in caries of the cervical vertebrae, but in the inflammation of the lymph glands which are imbedded in the posterior pharyngeal wall. Of 204 cases analyzed, Bokai placed in this class, in contradistinction to only 7 cases secondary to caries of the vertebrae. Children especially prone to inflammations of the lymph system. Cervical lymphadenitis is common among them. Frequently it is tuberculous, but often not, and usually the acute variety whether suppurative or non-suppurative, results from infection previously existing tonsillitis. So also with retro-pharyngeal abscess, it is most reasonable to regard it as a secondary infection of the pharyngeal lymphatics from inflammation of exposed and associated muco-lymphoid glands, like the faucial and retro-pharyngeal tonsils. In rare instances the source of infection may be rhinitis communicated through nasal lymph channels or, still more rarely, a suppurative otitis, but as previously mentioned, fo-

lous and suppurative forms of tonsillitis, as well as those forms of tonsillitis and pharyngitis which are symptomatic of the exanthemata, may reasonably be regarded as the most frequent causes of retro pharyngeal lymphadenitis.

Whatever the source of infection, the initial stage of retro pharyngeal abscess is retro pharyngeal lymphadenitis, and moreover the lymphadenitis may be of a non-suppurative type, or the disease become arrested in this stage, undergoing resolution without the formation of an abscess. This simple lymphadenitis has been but rarely observed in this country, but Bokai, in addition to 400 cases of abscess, mentions 112 cases of simple retro-pharyngeal lymphadenitis as having passed under his observation in the Pester Kinderspital.

I would report the following instructive case.

A female babe 4 months of age, was convalescing from acute folliculous tonsillitis, probably of the variety which I have elsewhere described, under the name of infectious pseudo-membranous tonsillitis which was prevalent at that time, and which in several other cases, under my observation had occasioned pronounced cervical lymphadenitis. As the tonsillitis subsided dyspnea commenced and increased for two weeks, at which time I saw the case in consultation through the courtesy of Dr E J Kuh. Suffocation was imminent, cyanosis marked, respiration irregularly stertorous and only possible when the child was held in a certain position. The voice was unimpaired and inspection of the fauces negative, but careful palpation disclosed a hard tumor which projected from the posterior pharyngeal wall in the median line extending downward sufficiently to press upon the opening of the larynx. I made three punctures into this tumor but failed to evacuate pus and being otherwise satisfied by its hardness to the touch and the absence of fluctuation that it was not an abscess but a case of simple lymphadenitis, tracheotomy was advised. The recovery which ensued from this operation in a child so young as 4 months, is to be largely attributed to the skilful manner in which Dr L L McArthur opened the trachea without an unnecessarily large external wound, without delay and with antiseptic precautions. The tumor underwent gradual resolution without suppuration or at least there was no perceptible discharge of pus in any quantity. At the end of two weeks the tracheotomy tube was withdrawn and the child recovered.

The successful management of such a case would depend first on a correct diagnosis. Dyspnea is the most prominent symptom, the cause of which must be precisely determined. It is easy to exclude diphtheria by inspection of the fauces, and equally easy to exclude both spasmodic and membranous croup by the presence of an unimpaired voice, but I have seen identical symptoms of suffocation produced by a large collection of "adenoids" in the naso pharynx, which by pushing the velum palati downward and forward, caused it to impinge on the dorsum lingue and thus impede respiration. This condition can be excluded and the positive diagnosis of retro pharyngeal lymphadenitis made not by inspection, but by palpation. Examination deliberately made by the finger of each and every part of the pharynx and naso pharynx will afford much more exact information in such cases than mere inspection of the fauces.

Having located the tumor in the posterior wall of the pharynx, the presence or absence of pus may be determined by a puncture or two or three punctures carefully made by a bistoury. The exact position of the tumor varies in different cases, it may be high or low, in the median line or to one side. "McClellan's Regional Anatomy" locates one retro-pharyngeal lymphatic gland opposite the second cervical vertebra, but it is likely that it may deviate from this position, and that others would be located by

close anatomic study. An inflammation commenced by infection of a lymphatic gland would be likely to involve, also, the surrounding connective tissue. When located low down in the laryngo pharynx a comparatively small swelling may speedily occasion suffocative symptoms.

This paper includes within its scope the treatment only of retro-pharyngeal lymphadenitis not that of abscess, and apart from general measures the only symptom of the former affection likely to demand interference is dyspnea. Having made preliminary punctures to exclude a possible collection of pus, the question of tracheotomy must be considered. Intubation is out of the question, for the same swelling which stops the glottis would stop the orifice of the tube.

Bokai finds that tracheotomy is rarely necessary, resolution usually taking place in from five to seven days before suffocation becomes imminent. Nevertheless, Bokai reports a case similar to the one herewith detailed in which he performed tracheotomy. It was a child 8 months old, the posterior wall of the pharynx showed diffuse hard swelling without fluctuation, and a deep incision into the mass had yielded no pus. Resolution occurred without suppuration, the tube was withdrawn at the end of three days and the child recovered.

Unlike diphtheritic croup, early age is no contra-indication to the operation of tracheotomy in retro-pharyngeal lymphadenitis, for herewith are recited two cases under 1 year of age, my own of 4 months and Bokai's of 8 months, in which recovery occurred. Good judgment would therefore indicate that whenever dyspnea has advanced to the verge of suffocation, or even to threatened exhaustion of the patient, tracheotomy should promptly be performed.

VALUE OF ANTROSCOPY (ENDOSCOPY OF THE ANTRUM OF HIGHMORE)

Read in the Section on Laryngology and Otology at the Forty fifth Annual Meeting of the American Medical Association held at San Francisco June 5-8 1894.

BY HENRY LEWIS WAGNER, M D
SAN FRANCISCO CAL

I am convinced that we all are not always successful in the treatment of empyema of the antrum of Highmore and consequently we have been obliged to perform a second and more extensive operation. This has taught us the real nature of the disease, and how to thoroughly eliminate the existing trouble. All this may be avoided by a thorough ocular inspection of the maxillary sinus by the antroscope—the endoscope of the antrum.

Two years ago I demonstrated this in a crude state to the San Francisco County Medical Society. I do not need to explain the details of the principles of the endoscope, which are used for illuminating closed cavities, as you are all familiar with them. The endoscope for the antrum is intended to be used after perforating through the alveolar process or through the fossa canina. It can also be used with the proper curvature through an intranasal opening. The latter method I have abandoned during the last years.

The cases in which the antroscope has proven to me to be useful, were

1. Jahrbuch für Kinderheilkunde Band xxxiii Heft 3 p 260 Amerl
can Journal Medical Sciences p 120 July, 1892.
2. Loc. cit

1 To determine the presence of a drainage tube in the cavity, which was said to be missing

2 Where a polypus near the hiatus semilunaris could be located, which had kept up a sero-purulent discharge

3 Where by a series of bony septa the pus was retained

4 To ascertain the differential diagnosis of angina, where suddenly a severe hemorrhage took place from the antral cavity

5 To facilitate the thorough scraping of the necrosed bone by means of a bent curette—introducing first the curette and then the antroscope

NEW INSTRUMENTS

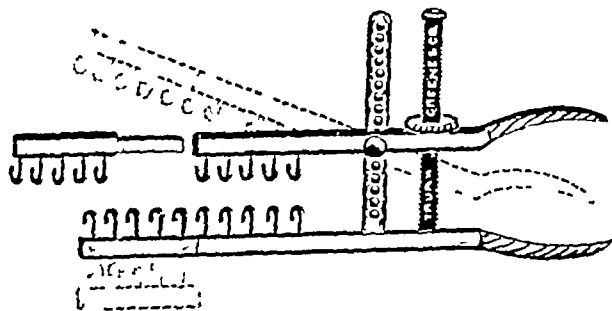
Read in the Section on Laryngology and Otology at the Forty-fifth Annual Meeting of the American Medical Association held at San Francisco, June 5-8, 1901

BY SETH SCOTT BISHOP, M.D.

PROFESSOR OF OTOTOLOGY IN THE POST GRADUATE MEDICAL SCHOOL AND HOSPITAL, CHICAGO, ILL.

DOUBLE RETRACTORS

The double retractors take the place of an assistant in keeping the soft tissues out of the way of the operator, and in controlling the hemorrhage during mastoid and other operations of like magnitude. The retractors consist of two shafts, each armed with a series of hooks that can be brought together and interlocked for insertion into the incision, when they can be separated and fixed at any desirable point up to two inches apart. After they have been drawn apart as far as may be required, the thumb screw on the fixation bar next to the hooks should be screwed down firmly into the bar, the handles should be pressed a little together until the tissues are well stretched as the distal ends of the retractors separate, when the thumb nut on the thread bar should be turned down against the movable handle.



If the instrument is properly adjusted the tissues can not slip out of its jaws and their pressure on the stretched lips of the wound reduces the hemorrhage to a minimum. In one operation these hooks proved more effective than five artery forceps.

The following arrangement renders these retractors equally useful in the smallest and the largest mastoid operations. The outer half of the shaft of

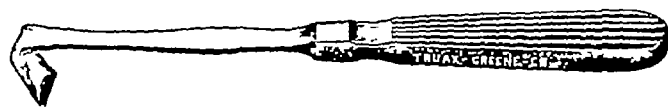
hooks can be slipped out of the inner half, leaving the retractors only an inch long. Replacing the adjustable series of hooks makes them two inches long, and by drawing these adjustable hooks outward one-half inch you can lengthen the hooks to two and one-half inches. This has the effect, when the instrument is in position in a large wound, of making an opening two inches to three and one half

inches wide, by three or more inches long through which to work. However the opening can be made as small as one wishes, and the capacity of the instrument is far beyond what we usually require in operations on the skull, but I have had it made so as to be of service in other and more extensive operations, since its size in no way impairs its efficiency in mastoid cases.

When the adjustable parts of the hooks are removed for small operations the openings in the permanent hook-shafts, into which the adjustable hooks fit, may be securely sealed by a bit of beeswax to prevent the entrance of blood, etc. After being used, this wax will run out on the application of a little heat. A drop of oil should then be put in the same openings to prevent corrosion, or sticking of the adjustable shank.

A PERIOSTEUM SEPARATOR, RETRACTOR AND CURETTE

This hoe-shaped device overcomes a serious objection to the misnamed periosteotomes we have been using. Indeed these instruments should not be *tomes* at all. They should not cut the membrane, but should lift it from the bone in continuity, so as to carefully preserve its integrity.



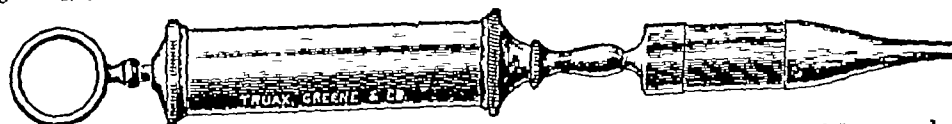
The old periosteotomes put the operator at a disadvantage by necessitating an unnatural play of his muscles. With a pushing motion one has not perfect control of the movements of the instrument and it is likely to slip and cut where it is not desirable to wound. In the use of this kind of a lifter the motion is one of drawing or pulling toward one's self so that the muscles brought into play are, together with the instrument, under easy control—on the same principle as the farmer's use of his hoe, after which it is patterned.

As the separator serves the purpose, not only of detaching the periosteum, but of retracting the loosened tissues, or of curetting necrosed bone, it may be said to constitute three instruments in one.

EAR ASPIRATOR

This instrument is for the purpose of evacuating, or aspirating, the middle ear and adjacent cavities of pus. It consists of an improved air pump and adjustable glass air chamber that fits into the external auditory canal by means of a soft rubber covered tip.

A few gentle tractions of the piston will draw out the discharges from the attic and deeper recesses after the most thorough ordinary methods have proved futile. Not enough force should be employed to cause actual pain or much sanguineous effusion. Yet a slight show of hemorrhage is not disadvantageous, for the parts are stimulated and thoroughly washed of discharge.



The pump is serviceable in chronic cases of caries of the attic, aditus, and antrum in order to obtain complete evacuation and cleansing as the first step in every treatment.

These instruments were made for me by Messrs Chas. Truax, Greene & Co., of Chicago.

Columbus Memorial Building

THE CARE OF PREGNANT WOMEN

Read before the Association of American Obstetricians and Gynecologists 1894

BY WILLIAM B. DEWEES, A. M., M. D.

SALINA, KAN.

It is a stirring conviction with many reputable, earnest and progressive obstetricians, that the time has come when we must disclose our power to the world by increasing the usefulness of our labors, and begin to use it for the prevention, as well as for the alleviation, of the sufferings of pregnant women, as has not yet been done, or else get out of the way. In such an event, deliverance will come to this class of suffering women from another source, but woe to those of us who are found at ease with the assumption of this responsibility. The responsibility is tremendous, but the obstetrician can not evade it. He may selfishly shirk it, but it is absolutely out of his province to shift it. That it is our most sacred duty to purpose awakening the profession by arousing one another, until each and every member is fully astir in the line of progression—the indifference of which is the greatest hindrance the medical profession has to contend with to-day—is the writer's hope and belief. The specialist just now is being put to the sharpest test ever known in his history. Whether we as obstetricians will endure it, and turn about to accept it as the greatest opportunity the centuries have brought us, depends upon our readiness to hear and heed the voice of Nature, so very clear and distinct to day. These are matters of great importance to us, and this is a meet place and time to bring them to notice and to consider them. No one can read, listen, and observe with intelligence now, and not feel that we are living in one of the most marvelous and, for the true-hearted, progressive practitioners, one of the most responsible epochs the world has ever known. That the medical profession is not fully awake to the needs of the hour, as she should be is sadly true. Far be it from me to depreciate in the least degree, the noble work done and the glorious advances made by our profession for human physical betterment. She is unmatched by all other organizations combined in this respect. But, it must be remembered that she should be. It is just this that gives significance to the contrast between what she has done and should have done. She has need to be taught again her first duty. To preserve the human body sound which begin the first principles of the oracles of God.

Let us recall the three great and noble objects of medical science, namely 1, to preserve health, 2, to allay suffering, and, 3, to banish disease. The more immediate duties of the obstetrician are to preserve life, to mitigate pain, and to expel disease, amid the natural or accidental difficulties to which pregnant and parturient women are constantly exposed. The study of the diseases and difficulties encountered with a state of pregnancy and parturition, though highly interesting and having at all times attracted the greatest attention of physicians is, however, exceedingly difficult. This circumstance probably atones for the fact that our obstetrical literature still continues to be full of errors. By consulting the best practical writers, missionaries, and travelers, the revelation comes that it is among the higher classes in the more civilized countries—Europe and North America—that women suffer the greater in both pregnancy and parturition, but that the women of the peasantry and the savage are comparatively

exempt. We may therefore very naturally and wisely conclude that it is unnatural for civilized woman to suffer so universally as she does to-day; and that civilization in disobedience to the law of Nature, is the true cause of her present suffering during gestation and childbirth. Thus we may

"Find out the cause of this effect,
Or rather say the cause of this defect,
For this effect defective comes by cause."

It is ignorance, as well as carelessness and viciousness, that has brought about the existing abnormal condition of civilized women. Thus, through unwholesome civilization have come fixed habits of excesses, and if excesses must be indulged, evil consequences will follow and must be endured, for Nature's law would have to be changed before they could be either prevented or banished by any method of treatment. It follows, then, as a natural consequence that to prevent these sufferings will depend, not so much on treatment by application or administration of therapeutic agents, as on the successful education and training of these women, so that they will learn to know how to—and will actually—cultivate the necessary self-discipline requisite to enable them to prevent the continuous irritation from excesses in their modes and habits of life. If the writer's observations have been correct, and unprejudiced, it seems that a revival of learning in obstetric science must needs be instituted among us, destined to find the true cause or causes of the unnatural and needless suffering of civilized women. The progress of obstetrics in the immediate future must be made through the knowledge that will be wrought out by the devotees of biology. Thus, we shall find our way, on positive ground back through the morphology of organs, tissues, cells and blood, to a clear comprehension of the origin of vital activity in protoplasm and the papulum which sustains it. This is the only way open for us into the primitive arcana of Nature, if we would have the wisdom essential to intelligently inculcate that regimen which will successfully prevent the needless sufferings of pregnant and parturient women.

The future distinction of obstetric science can obtain only by an advanced study of human biology. When this truth is propounded, there opens before the thinking mind a vista so transcending all ordinary limitations of obstetrical knowledge, as requires such genius and expansion of the mental eye in order to embrace it in its simplicity as scarce yet obtains. It remains, then, for our guild, to endeavor by rigid scientific investigation through advanced biological studies, to make patent the causes of the sufferings of pregnant and parturient women, and to determine exact measures for emasculating these causes, or for neutralizing their effects whenever they have eluded detection or escaped emasculation. Thus, by directing our studies chiefly in relation to etiology and diagnosis, and having learned to know the true cause it will become easy to intelligently establish prevention, while, with a correct diagnosis, treatment will become easy.

I verily believe that, the next coming great advance in this, our special branch of medical science, will be in our bringing home to the general practitioner the fact, that the diseases peculiar to women during pregnancy and parturition are very largely preventable. To make him feel his responsibility, both as to their production after the present

generally prevailing methods of practice, and also as the possibilities of their prevention after improved methods. The family physician must be fully roused to the conscious realization of the fact that lies within his power very largely, to prevent many of the diseases among the women of the families entrusted to his care. When this obtains, his moral obligation will impel him to promptly do his full duty, by giving adequate instructions concerning the ill effects of improper posture, dress, food, drink, and erroneous habits of living, including the non forbearance of indiscriminate, excessive and impure sensual indulgences. Then it will come to pass that the wholesome forbearance of coitus during the entire period of gestation, the puerperium, and three months hereafter, will be insisted upon, together with correct posturing and dressing, proper food and drink, and healthful habits of living. Aseptic accouchments will be faithfully and efficiently practiced, lacerations of the cervix and perineum will be more frequently prevented, and when they do unavoidably occur will be early repaired, rectal and vesical troubles will be corrected in due season, gonorrhea and syphilis will be also more frequently prevented, and when encountered will be more vigorously treated and kept under surveillance until all discharge and sequelæ are arrested. And, what will be of far greater benefit to mankind, there will be given the requisite attention to the growing girls, with special care about the years of puberty, that more vigorous bodies and more normal reproductive organs be developed. A positive, clear and convincing protest will be made against the present unwholesome state of society which is now urging our girls on to degree of emasculated physical corruption, by uxorious indolence, deleterious dress and sensual excess, and superbly fits them to live in communities by themselves—in women's hotels—whereby they become ruined for wisely companionship and sturdy maternity. When that day comes—as I verily believe it to be within the province of our intelligence to successfully inculcate—then, and not until then may we hope to find that the prevalence of diseases among civilized women will cease to be a reproach to preventive medicine. Then will authors and teachers find it necessary to discontinue defining woman as, "an animal with a constipated bowel and a pain in her side."

But as yet, at present we are compelled to meet the situation as we find it. The condition of the suffering of the civilized women of the present generation, however grossly unnatural it has been cultivated, must needs have our most careful attention and require our wisest judgment, lest we fail to institute proper treatment for their relief. In the care of pregnant women much of great importance devolves upon the obstetrician, with regard to safely conducting the mother through the period of gestation, parturition, and the puerperium, the preservation of the life and healthful condition of the child, and his own reputation. The diseases of pregnancy unquestionably is a field only partially and imperfectly explored. When we take into consideration how much of accident might be avoided and how much real good done to this class of suffering women by proper care, it places us at a loss to account for the fact that obstetricians have hitherto neglected their duties, by signally manifesting a want of endeavor to promote the advance of obstetric science,

in a direction quite worthy of the profoundest investigation. It is a noteworthy fact that the conditions which beget the disorders of pregnancy are not thoroughly treated by any of the standard works upon obstetrics. We are so busy thinking, discussing, defending, inquiring, practicing and teaching, about the better modes of alleviating human suffering that we have no time and no leisure of mind for quiet contemplation, to allow the exercise of the intellect to feed upon Nature's truth, without which it is impossible to obtain a clear knowledge of the real causes and how to successfully prevent them. Communion with Nature was never more needful than now. Feverish activity rules all spheres of life. Medical effort is multiplied and systematized beyond all precedent. And all these things make calm fellowship with Nature hard to compass. We can conquer Nature only by obeying her laws, and in order to obey those laws we must first learn what they are.

For the convenience of our consideration, the paramount duties of the obstetrician in the study and care of pregnant women may be classified as follows:

- 1 To discover if the patient be actually pregnant
- 2 To determine positively if the impregnation be uterine or normal, as contra-distinguished from tubal, abdominal, or abnormal pregnancy
- 3 To carefully note the pregnant woman's history, including her age, primiparity or multiparity, environments, station in life, general condition of health, period of gestation, as well as her dress, food, drink, and habits of life. To make repeated examinations of the urine and ascertain the temperature, from the time pregnancy is established to the termination of gestation

4 To make a physical examination for the purpose of accurately determining the diameters of the pelvic straits, the symmetry and size of the bony outlet, the integrity, condition, and position of the vagina, uterus, and other inter-pelvic viscera, and adjacent structures, the state of the abdominal muscles, the presence or absence of hernia, varicose veins, tumors, etc., the shape, size and condition of the breasts and nipples, the condition of the heart, lungs, mind, stomach, bowels, etc.

- 5 To observe the state of the fetus, its strength and viability, as well as the implantation of the placenta

These must all be included in the search for intelligent guidance, as to how the proper management of the pregnant woman should be conducted. With regard to the first, all experienced observers have found that psychologic phenomena often call for an intimate study and wise differentiation of every form of hallucination, delusion, illusion, as well as the delirium of cerebral hyperemia, or the frenzy of the maniac, from toxemia and eccentric irritabilities. And, that such abnormal conditions frequently exist unrecognized, and so continue until they eventually establish their peculiar fixed impress upon the mind and nervous system. Thus when the paranoiac woman simulated pregnancy, and even parturition, she deceived the better judgment of some of the most skilled obstetricians. Pseudocyesis and pseudotocia are recognized abnormal conditions, which so closely resemble the normal condition of pregnancy, and the beginning of parturition, as to demand our highest discriminative faculties in arriving at an intelligently correct diagnosis.

As to the second, it remains a simple self-evident

fact that it should always be positively determined whether the impregnation be uterine or extra-uterine. How to intelligently differentiate normal or uterine from abdominal pregnancy, time forbids to discuss fully in this connection, suffice to say that it may be made by careful palpation of the abdominal parietes, together with vaginal and rectal digital examination and proper posturing. Paracysis and interstitial or cornuate fetation are purposely omitted, as such accidents can scarce be diagnosed at a sufficiently early period of gestation to be of actual benefit. Abnormal implantation of the fetus constitutes a most important class of cases with which the accoucheur has to contend. Extra uterine or tubal pregnancy is rarely suspected until after certain dangerous and distinct symptoms are manifested, chiefly because no opportunity is had to discover it. These symptoms are such as follow rupture of the cyst containing the embryo with intra-pelvic, or interperitoneal hemorrhage and shock, as sudden seizure of violent agonizing pain in the lower abdomen, fainting, pale, or anemic surface of the body, cold clammy skin, rapid and almost imperceptible pulse, sighing respiration, etc. Whenever extra-uterine fetation is discovered, either before or after rupture of the cyst, the question of treatment is a very grave one. But experience teaches us that this truly marvelous and murderous condition admits of only one line of action to be followed with any degree of ultimate safety to the life of the pregnant woman. Section must be done. No other line of treatment that has been advanced is so rational and certain in the ultimate saving of life as abdominal section. The perfected technique as we have it to day, makes it a safe procedure. We must, however, bear well in mind what experience also teaches us, namely that when the placenta is found still in the tubal sac it is best to enucleate and remove all—the tubal sac and ovary, but that whenever the placenta is found partially or wholly out of the tubal sac and adherent to the peritoneum, bowel, etc., and still alive, the very best that can be done is to let it alone—to keep hands off. In these cases the ultimate safety of the patient lies in removal of the fetus, cleaning out the peritoneum of blood clots and all other debris, ligating the bleeding vessels, pack with iodoform gauze, and treat as an open wound, thus allowing the placenta to come away in due course of time by suppuration. We can thus guard against sepsis by drainage and cleanse out the cavity as fast as putrescence develops. My most venerable friend and classmate at the University of Pennsylvania, Dr Joseph Price, than whose experience in these cases there is none more extended and successful, and consequently he deserves to be accepted as reliable authority, says "All other lines of treatment that have been advocated fall into insignificance with this manner of managing these cases." He also claims this is a summer disease or accident, being in his experience almost always found only during the summer months, when May comes he looks for extra-uterine pregnancy cases.

The third brings us thoughtfully to consider the influences of those conditions which have been hitherto so grossly neglected, but which demand a decided and determined prophylaxis. The unprecedentedly rapid progress of civilization in disobedience to the law of Nature, as evidenced by the accumulation of wealth on one hand and extreme poverty on the other, the forcing or cramming system of intellectual edu-

cation with lack of requisite physical training, the custom of unwholesome food and drink, deteriorating fashions of dress and habit of society, unbridled sensual indulgences, and the woful endeavor to equalize the duties of women with those of men, have within the last generation very much deteriorated the child-bearing capacity of civilized women. True, the standard of female beauty is increasing, and decided intellectual growth manifested by women in all departments of science and art, but, in the same ratio do we find the remote dangers as well as the immediate accidents of gestation and child-birth have increased. Hence, we find that flaccidity of the abdominal, spinal and pelvic supports, chronicity of the shattered exotic nervous systems, abnormal state of the ovaries, subinvolution of the uterus, etc., have marvelously increased in later years, notwithstanding the much vaunted so called hygienic advances in improved habits of living and reform in dress. These unnatural conditions are most prevalent among the residents of our cities, and increase from bad to worse as we go from the smaller to the larger and more crowded marts in civilization. They are as prolific sources of chloro-anemias, trophic-nerve paresis, hydremias, hyperemias, and prostrations during pregnancy among the women of wealth, ultra-refinement and excessive culture, as are those women who are subjected to the privations and hardships of extreme poverty. The remarkable fact in history being that there has been a proportionate increase of these conditions among the women of these two extremes of life, whereas the reverse holds good with women who belong to a middle class and are comparatively free from the unnatural behests of modern civilized society, or of irremediable poverty. The pregnant woman whose parentage, as well as herself, has been untrammelled with the deleterious impress of either of these two extremes in life, will terminate her gestation and parturition with much less suffering, and more healthful to herself and her offspring than will her more unfortunate sister who comes from a stock reared in the excesses of indolence, luxury or poverty. The pregnancies of too early, as well as of too late, marriages are alike to be apprehended as fraught with unnatural conditions. The girl who enters the matrimonial state at the nubile period, from 18 to 20 years of age, will approach the marriage couch with a much better prospect of whole some fruition, healthy to herself and offspring, than will she who enters the nuptial relationship earlier under the turbulence of puberty, immature physical development, and illy formulated morale, amid the storm waves of exalted nerve tensions superinduced by over-taxed educational training, and undue stress of unwholesome fashion and society, or, than she who marries late, say after 28 years of age, who still retains all the productive elements but which have been debilitated by repeated disappointments, not leave her with the elasticity and resiliency of youth—the regulating factors in all growths—defective and the newly stimulated energies of a life somewhat wasted are revived in a physical organism unfitted for the reproductive process. The ovaries and uterus may properly perform their respective normal functions from puberty to the menopause, but it must be remembered that an opportune impregnation is requisite to preserve woman, if not rosy and plump, at least elastic and buoyant, that her maternity may be exempt from pelvic indolence, so typically manifested

by the flattened chest, the wrinkled face, and some fixed cultivated hobby—to save the rosy-cheeked maiden of 20 from shriveling into the parchment-skinned spinster of 40, by timely desuetude of celibacy. The primiparity or multiparity of the patient also demands its quota of consideration in the etiology of the diseases of pregnancy. The influences of heritage, personal habits, station in life and environments, usually make up the requisite field for the consideration in the primipara, whereas, in the multipara we find it essential to also look well after such conditions as the subinvolution of the uterus, lacerations of the cervix or perineum, tumors, and defective pelvic, abdominal and spinal support, as they often lead to abortion or to direct dangers during parturition.

The thoughtful obstetrician will, with inexorable discipline, advise his patient as to the requisite *regime*. The consciousness of his full duty will impel him to insist upon

1 Absolute, regular hours and wholesome environment

2 Plain but nutritious and wholesome food and drink, being principally composed of fresh lean meats, fresh fruits, pure milk, and distilled water

3 A proper amount of exercise, by walking or light labor on foot and maintaining the correct erect posture. Whenever infirmity forbids such exercise, recourse should be had to massage, and as much time passed in the open air as is advisable under such unfortunate circumstances. Rest in the recumbent posture after meals and fatiguing efforts, with not less than ten hours sleep out of every twenty four

4 An open condition of the bowels and skin, which is to be chiefly maintained by proper diet, exercise and bathing, the wearing of flannels, warm low-heeled shoes and loose garments, and, in rare cases, the proper use of laxatives, and hot water enemias

It, however, not unfrequently falls to our lot to have placed under our care pregnant women who belong to the poorer and less favored class, exposed to too much labor, privation of proper food, dress, and shelter, with whom we find ourselves confronted with the question for bettering their diet, dress and habitation, which assumes such proportions that demand our assistance, not only as medical men, but also, as philanthropists. Fortunately the advantages which the many charities afford enable us to give this class much benefit in every way, save when mental anguish and nostalgia become irremediable factors, in the production of depressing influences which bar the healthful action of the resources at our command. With regard to repeated examinations of the urine and noting the temperature, it suffices to say that as is the urine and temperature so is, very largely, the prognosis and treatment prior to parturition. Urinalysis and thermometry are simple in detail, yet how prolific of averting the culmination of conditions very hazardous to both mother and child. Conditions which otherwise are frequently discovered only by the appearance of anasarca of the inferior extremities, edema of the face or lungs, or a seizure of eclampsia, after which attention is given to these searching steps, but often too late to be of any value in devising a prophylaxis. This very naturally raises the question, Is there a prophylaxis for the albuminuria of parenchymatous nephritis during pregnancy and consequently the avoidance of eclampsia prior to or during parturition?

The experience of an earnest rational endeavor in a limited number of cases warrants the opinion that very much may be done in this direction. So, also, with regard to the prophylaxis of parturial sepsis, which is a problem of equally great magnitude. True, sporadic cases of so-called puerperal fever (parturial sepsis) as well as of eclampsia, etc., ensue in spite of all possible efforts, but it is the writer's firm conviction that these murderous complications can and will be prevented by timely precaution and by banishing every factor in their production. Preparatory hygienic regulations, antisepsis and quarantining will as surely save the mothers from these complications as absolute cleanliness at parturition will save the child from ophthalmia neonatorum.

In proof of this, it is needless to confine reference to personal experience in private practice, for in addition we find the maternities likewise furnish abundant evidence in support of this declaration. Here we may cite, as the most striking example, the indefatigable labors of our eminent Fellow, Dr. Joseph Price, in his connection with the Preston Retreat at Philadelphia, whereby both that institution and himself have become renowned, in establishing this fact beyond all doubt or cavil, that these complications can be prevented.

A word with regard to the term, puerperal fever, which is misleading and fails to express the condition it is intended to imply. It should therefore be expunged from our literature and be replaced by the proper term, *parturial sepsis*. Parturial sepsis is a surgical sepsis, arising from the conditions in which women are found during the extrusion of the uterine contents, similar to those during surgical procedures. The same classes of septic infection and septic poisoning occur in the non-pregnant state, during operations upon the pelvic viscera done without due regard to absolute cleanliness, and in hospitals where patients are crowded together with want of proper sanitation. Facts, as numerous as can be collected in any department of medicine, prove conclusively that the infection and contagion of sepsis, non-specific and variable as they are, can be conveyed by the nurse or attendant as well as by the accoucheur, and thus may likewise poison patient after patient until they also, as well as the accoucheur, either discontinue their labors or subject themselves to the most rigid discipline of absolute cleanliness and sanitation. When this prophylaxis obtains, the will parturial blood poisoning cease to be among the cases intrusted to their care, and they will find themselves rewarded with a mortality of *nil* from sepsis. It is very clear to the writer that the more carefully we study and practice obstetrics on sound surgical principles, the better success will we obtain. This, above all else, will secure a thorough preparation of the vagina, that the child may make its exit into the world through a clean channel, which will insure safety to both mother and child. And again, if the forceps or the hand should be required, if laceration of the cervix or perineum should occur, the patient will then have the healthful benefit of the same preparation as is given for surgical procedure. A word also with regard to finding ourselves summoned during the latter period of pregnancy, or at the approach of parturition, to attend one who is suffering from or threatened with eclampsia, and the case being a plethoric subject I should feel it as much my duty to bleed, as I should to clean out the endometrium.

whenever parturial sepsis was found to be due to the retention of infected decomposing portions of the secundines. While, if the patient under like condition belonged to the more rare class of anemic cases, I should hope to happily correct the disorder by chloroform, hot water, the hypodermatic use of morphin and atropin, veratrum viride, etc.

Under the fourth head of the subdivision of this subject, it may be remarked that pelvimetry is an equally essential requisite with urinalysis and thermometry. Deviations from the normal symmetrical pelvis, the diameters of the pelvic straits and the inclination of the bony excavation, exercise marked influences in proportion to the degree that they exist. In an abnormally broad capacious pelvis, with obliquity lessened and supports relaxed, we find that in the earlier period of gestation the tendency is to misplacement of the gravid uterus, either forward or backward and downward, from gravitation in consequence of increased weight. These abnormal uterine positions are usually manifested early by bladder or rectal difficulties with bearing down or pressure in the pelvis, and a dragging sensation from the lumbo-sacral region, etc. The functions of the intra-pelvic nerves, blood vessels and lymphatics being interfered with, we find in a proportionate degree that there is produced a stasis in the venous, vaginal, uterine and rectal plexuses, and consequent dilation of both these vessels and the lymphatics. Thus we have produced a hyperemic state of the entire pelvic circulation resulting in edema primarily, followed by ecchymosa of the peri-vascular, peri-lymphatic, and other cellular tissues, to be succeeded later by varix of the vulval, vaginal, uterine and hemorrhoidal venous plexuses, and consequent trophic nerve changes. It is a noteworthy fact that in these cases there is almost invariably found a want of the correct erect posture. The woman, herself, unconsciously increases the aforesaid difficulties by assuming a more or less stooping posture, in her endeavor to make herself comfortable from the dragging sensation below. Thus we find the abdominal and spinal muscles relaxed, the lumbo-sacral spine receding behind its normal axis-perpendicular with ear, shoulder, hip and ankle—the weight of the superior trunk gravitating back of this normal axis and falling perpendicularly behind the heads of the femurs, whereby the pelvis is swung upon the femur heads from an oblique to a more or less transverse position, while the superincumbent weight of the abdominal and thoracic viscera, instead of being directed forward, by the normally advancing lumbo-sacral spine and supported upon the pubes and lower portion of the abdominal muscles, now falls directly within the basin of the pelvis, exerting its injurious effects upon the contents thereof. The real causes of these conditions being thus clearly revealed, the treatment becomes very simple. This consists in first reestablishing and maintaining the correct erect posture, thereby securing the normal obliquity of the pelvis by advancing the lumbo sacral spine in its normal axis of the body. The superincumbent weight of the abdominal and thoracic viscera is then again directed to normally gravitate upon the pubes and lower border of the abdominal muscles, and at the same time there is afforded the normal shelter to the pelvic contents below and behind the promontory of the sacrum. Experience has established the fact that the correct erect posture in these cases is

secured and maintained the more effectually by the virtue of proper external support. The external support which fulfills this purpose the better is fully detailed by the writer in a paper read before the International Congress of Gynecology and Obstetrics, at Brussels, Belgium, September, 1893.

The normal equipoise of the superior trunk upon the lumbo-sacral spinal axis having been thus restored, whereby the evil influences from the weight above are a prime causative factor in intra-pelvic disturbances are happily overcome, there remains but attention to be given to the insignificant weight of the misplaced pregnant womb itself. This is usually easily corrected, when free from adhesions, by proper padding, and afterward maintained by proper internal support with some pessary suitably fitted to the condition.

When, on the other hand, the pelvis is abnormally distorted or contracted, the question of how best to conduct the gravidity has been even a more unending source of discussion than the preceding conditions. Here pelvimetry furnishes us the chief guidance. But before we progress further, let us first break loose from the traditional shackles of our predecessors and their authority, which have too long retarded freedom of thought to such a degree that who dared to doubt met but derision. I refer chiefly to the murderous practice of inducing premature delivery and embryocia in this condition. In the present light of science these procedures have no place in the obstetric art in connection with a viable fetus. They simply deserve to be mentioned in the past, they may be the more effectually relegated to the past. This leaves us to choose principally between but two procedures whenever we find the pelvis distorted or contracted that it precludes all probability to deliver the living child, namely, symphysectomy and Cæsarean section. Without specifically considering all the points of this very serious condition, which fortunately for us, is, comparatively speaking, very rarely encountered in the native born American women—I would unhesitatingly advise against the termination of gestation, looking more hopefully to ultimately saving the life of both mother and child by resorting to one of the aforesaid operations. It is to be remembered, however, that it is the duty of every intelligent obstetrician to become thoroughly familiarized with the indications as well as the technique of both these operations, neither of which are to be lightly undertaken by any one who does not possess the requisite skill, both in the obstetric art and abdominal surgery.

With regard to the proper selection of these two operations, I can do no better than quote my friend Dr. Barton Cooke Hirst, of Philadelphia, who is an acknowledged authority on the obstetric advances in his address before the Washington Obstetrical and Gynecological Society Nov 17, 1893, and published in the *Medical News* of Philadelphia, Dec 2, 1893. "It is obvious therefore, that symphysectomy can be preferred to Cæsarean section on the one ground alone—that it is less dangerous to the mother. A careful study of the results of symphysectomy and of Cæsarean section will show that the latter is, at the very least, twice as dangerous to the mother as the former. The mortality of the infants is not quite so great in deliveries by Cæsarean section. Moreover, by Cæsarean section the child is sure to be delivered easily

¹ This support is manufactured by the Natural Body Brace Company, Salina, Kan.

no matter what the size and shape of the pelvic canal may be and there is no necessity for that nice calculation in pelvimetry that is usually demanded before contemplated symphyseotomy, a calculation in which even the best of us may err. With my own experience of three perfectly uncomplicated recoveries from symphyseotomy, I feel disposed to regard this operation with more favor than version and extraction in a markedly contracted pelvis. The opposite error of opening the symphysis when the pelvis is too small to permit of delivery by this means is more difficult to escape. It will only occur in a pelvis with a conjugate of sixty-seven millimeters, or under a degree of contraction not often seen among us, and could we always be sure of the accuracy of our pelvic measurements, the mistake would pretty surely be avoided. I can imagine no more embarrassing predicament in obstetrics than a failure to extract the head after opening the symphysis. I should feel reluctant to operate again at the lowest limit for symphyseotomy unless I had the opportunity first to induce labor and artificially dilate the cervix some two or three weeks before term. In this case I should not hesitate to operate with a conjugate as low as sixty-five millimeters, and I believe delivery might be possible with a conjugate of only sixty millimeters. We may therefore be guided by the rule that at term, symphyseotomy is available only in cases where the conjugate measures over sixty-seven millimeters while if the conjugate is found to be sixty-seven millimeters or under, the only recourse to be had is Cæsarean section.

The destruction of the embryo is, however, not only warranted, but becomes a requisite under certain circumstances or conditions, such as the presence of large fibroids in the body of the uterus, or large tumors involving both the ovary and uterus, also cancers of the uterus, and in certain cases, of placenta previa. Placenta previa is another abnormal condition deserving attention. It properly belongs to that class of appalling accidents in the lying-in room, as eclampsia, post-partum hemorrhage, rupture of the womb in uterine pregnancy or of the cyst in tubal pregnancy, etc., which allow but time for action. Experience teaches that the proper use of forceps in these cases is to be preferred to the hand. The chief reason being that a narrow-bladed forceps can be introduced much earlier than the hand—requiring a dilation of only about one inch as compared with two and three-quarter inches for the hand—and, by grasping the head and bringing it down, tampon the placenta at once, whence time may be given sufficient to obtain full dilation and delivery with greatest possible safety to both mother and child. In these cases we may very properly follow the one line of action. As soon as the diagnosis of placenta previa is established our action must be prompt in evacuating the uterine contents. Hemorrhage is the danger signal. When this signal is given early, say prior to the sixth month, and the diagnosis established, we should proceed without any regard for the life of the child. When, however, this signal is given later, we should proceed as promptly, but with all possible endeavor to save the life of both mother and child. Whenever hemorrhage takes place in these cases it is Nature's signal to us that there is great danger ahead, that the condition is so serious as to endanger, if not to take the life of the mother at any moment. We can not

accept the situation only as serious, and must act promptly and effectually. The proper antiseptic precautions being observed with special reference to the vagina and the accoucher's hand, the patient being placed under anesthesia, introducing first the index finger into the cervix, dilating gradually until two fingers are successfully introduced, and, when sufficiently dilated to admit of the forceps, pass the fingers through the body of the placenta, rupturing the membranes and place the forceps on the head, bringing it down and tampon the placenta at once. When, as sometimes happens, the dilation is greater and the presentation abnormal or less favorable the accoucher had better resort to the passing of his hand and turning by Braxton-Hicks method and drawing the child into the cervix, and thus arresting the hemorrhage. The hemorrhage once checked, the subsequent delivery will be fully under control.

I have in this paper but sketched some of the salient and mooted points of the prophylaxis of the complications and needless sufferings of pregnancy, with no attempt at thoroughly elucidating or elaborating any one of them. Having no pet theory to promulgate and sustain at all hazards, no long list of cases and statistics with quotation and abstract from an exhaustive roll of writers has been made out. And, while universal conviction may not be with me on premises somewhat narrowly drawn, still I hope the attention of the Association may be drawn to their thorough consideration, that free discussion may evolve all the facts of this interesting subject and, finally, to find with many of you these facts received with favor and not only to maintain an excellent reputation, but also to increase in favor on closer acquaintance.

TRACHOMA OR GRANULAR LIDS

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Trachoma or granular lids (not granulated lids), ophthalmia militaris, ophthalmia Egyptiaca, trachoma verum, trachoma Arlti (Arlt), trachoma folliculare (Horner), trachoma mixtum (Stellwag), as it is variously styled has received much attention at the hands of ophthalmologists, and the literature upon the subject is so copious that it is almost an act of supererogation to attempt to present anything new and interesting upon the topic, but the dreadful ravages of the affection, and the fact that we are still far from a unity of views in regard to the disease, renders an attempt in this direction at least excusable. Not long since an article appeared in the JOURNAL OF THE AMERICAN MEDICAL ASSOCIATION from the pen of an eminent professor of ophthalmology in which the expression "granulated lids" was used, and while appreciating that by reason of the peculiar anatomic arrangement of the lids, granular and hypertrophic masses are distributed over the conjunctiva tarsi and transitional folds, yet we must insist that such a term should be reserved for the lathy as we are not familiar with anything like granulated lids, although such a term can be rightfully applied to sugar.

Before discussing the various forms of trachoma or granular lids, we will note a few historical data with respect to this affection. Relatively speaking, we may assert that it has only been within the last

century that trachoma has demanded much consideration at the hands of specialists, as it was only at the beginning of this century that the disease asserted itself as an epidemic among the European soldiers, hence the term *ophthalmia militaris*. Many concluded, by reason of its first appearance among the soldiery that it was a product of importation from Egypt, hence *ophthalmia Egyptiaca*, and the disease was laid at the door of Napoleon I upon his return from Egypt to Europe, it being presupposed that the disease was formerly confined to Egypt. But it subsequently transpired that it had been known in Europe since antiquity, and even to Celsus is accorded the honor of having described the condition, mentioning the characteristic roughness of the lids and the accompanying purulent discharge, and we find to-day a tendency to revert to the old-time methods of treatment by inducing a certain amount of inflammatory reaction which the ancients accomplished by means of friction with fig leaves and by specially devised instruments. So we must conclude from the foregoing that trachoma has existed from the most remote periods, and that the trachoma of to-day is nothing more nor less than modified acute trachoma of olden days, which was characterized by profuse purulency, and that the specific virus upon which its ultimate origin depends has become modified so that it is to day less virulent in its effects when transferred from one eye to another.

Character—Trachoma is a chronic, specific, infectious inflammatory affection of the conjunctiva accompanied by distinct purulency, and the name trachoma is derived from the Greek, signifying rough.

Symptoms—Individuals suffering with trachoma complain of watering of the eyes, agglutination of the lids, sensitiveness to light, pain and disturbance of sight, which are sometimes though not constant sources of annoyance. The eyes are less widely opened as a result of swelling and sensitiveness to light, and upon eversion of the lids we notice that the tarsal conjunctiva as well as that of the transitional fold is reddened and swollen, while at the same time there is a variable degree of roughness caused by hypertrophy which is of two characters in accordance with the different anatomic characters of the affected conjunctiva. The first form of hypertrophy is the appearance of the papillæ so styled, which are elevated patches here and there, newly formed over the conjunctival surface, which accordingly looks velvety, or the papillæ may become so large as to impart a coarse granular, nodular, or even a raspberry like aspect to the conjunctiva. Thickening of the conjunctiva is absent at this period, consequently the underlying meibomian glands gladden through the conjunctiva. This form of hypertrophy is limited to the conjunctiva tarsi and is styled papillary hypertrophy. This form of hypertrophy is peculiarly prone to attack the upper lids. Trachoma granules are characteristic of the second type of conjunctival hypertrophy. They are seen as grayish or yellowish-gray, roundish translucent gelatiniform bodies, and have been styled from their appearance the sago or frog-spawn excrescences, found chiefly in the transitional folds in such great abundance that when the lower lid is exposed to view the transitional fold projects as a thickened and rigid prominence. The trachomatous granules are sometimes disposed in rows like a string of beads,

while the trachoma granules are likewise shown to exist in the conjunctiva tarsi, they are less prominent and less clearly defined, owing to the close adherence of the conjunctiva to the tarsus in this situation, consequently we discern them, when the hypertrophic papillæ do not obscure them, as minute bright yellowish points, embedded deep down in the deeper structures. The microscope reveals that other parts of the conjunctiva of the eyeball, such as the semilunar fold, conjunctiva bulbi, etc., are not wholly exempt from the trachoma granules. This constitutes the so-called granular type of trachoma, so that we may justly classify trachoma into two classes 1, papillary trachoma, and 2, granular trachoma, but the distinction can not be so clearly and emphatically made as to enable us to say that this is a case of papillary trachoma, that a case of granular trachoma, inasmuch as frequently in the same case we find the two processes associated, and while such a classification would be quite scientific, yet such is not from a clinical standpoint of any particular importance as the treatment is essentially the same in either case.

Among the results of trachoma are pannus, lid distortion asserting itself under the form of entropion (the turning of the lid inward toward the eyeball), and ectropion the turning outward of the lid from the eyeball, symblepharon posterius and anterius, xerosis conjunctivæ, in which we have dry degenerated patches devoid of secretion here and there, and corneal opacities caused by cicatricial deposit following ulceration, trichiasis (abnormal disposition of the eyelashes) Madarosis and tylosis, baldness and thickening of the lid borders respectively are all disagreeable sequelæ of neglected and maltreated trachoma, and will receive mention under their proper heading during the discussion of the treatment.

Causation—We are, in the light of modern views relative to trachoma, able to say that the primal origin of the disease is discharge from a chronic blennorrhæa, which being transferred through the medium of the fingers, soiled linen, contaminated wash basins, etc. to the healthy conjunctival sac finds favorable soil for its development, after a variable incubative period a form of granular conjunctivitis or trachoma shows itself which depends for its severity, so far as its inflammatory tendencies are concerned, upon the character of the case from which it took its departure, as well as upon the character of the soil upon which it thus propagates. It is to Piringer that the credit is due of thus irrefutably deciding the origin as well as the subsequent features of the disease. He was able by inoculating eyes already blind, to determine that the disease is essentially dependent, notwithstanding all its variable characteristics, upon one infectious element, which by reason of the different anatomic conditions of the developing soil will assert itself sometimes in one form, sometimes under another, so that it leads us to conclude that possibly we shall finally view the variable types of granular conjunctivitis or trachoma, as well as other simpler types of conjunctival inflammation, as peculiar forms of inflammation or irritation consequent upon the special type of irritant.

With respect to the career of the affection, the hypertrophic elements and the associated discharge increase to a variable degree in different cases and then finally fade away, while cicatricial deposits replace

the hypertrophied areas, and consequently the conjunctival destruction and subsequent cicatricial contraction are directly dependent upon the number and size of the hypertrophic elements and the abundant secretion. So that with respect to the question of treatment two primal indications confront us, namely, reduce the size and number of the hypertrophic papillæ and check the secretion. And fortunately, while we are confronting one indication we are combating the other. Heretofore silver nitrate and copper sulphate have been largely utilized, both to reduce the enlarged papillæ and to diminish the discharge and favor the disappearance of the granulations, and these remedies are still employed and are regarded by many as all sufficient. The rule governing the use of silver nitrate and copper sulphate respectively is to apply silver nitrate when the secretion is profuse and irritative manifestations marked, while copper sulphate finds fitting application in cases of diminished secretion, and little or no irritation. Mercury bichlorid in concentrated solution, 1 to 500, was first applied after the so-called grattage in the clinic of Abadie, and by many is highly esteemed in the management of obstinate cases of trachoma. We must conclude, however, that all treatment should be directed towards assuring abject cleanliness, whether this be accomplished by simple ablution, or by means of antiseptic precautions. While the method of exciting an inflammatory reaction, either by means of medicaments, or by the more active method of jequirity infusion, as advocated by De Wecker (justly superseding the old-time method of inoculation with blennorrhæal pus) the methods of peritomy or syndectomy, or incising the conjunctiva around the cornea, scraping or scarifying the conjunctiva as advised by Gruening, are useful in certain cases. It has been my experience to find that these heroic measures have all been carried to extremes, to such an extent indeed that one of the most important indications in the treatment of trachoma or granular lids has been overlooked, namely, that the inflamed parts should be subjected to a period of rest, and any measure which fails to encompass this end utterly fails of its object. To institute this purpose, and at the same time to meet the other indications of resorption of the hypertrophic papillæ and granular masses combined with a proper degree of antiseptis, I was induced to apply insufflations of papoid (*carica papaya*), the new digestive ferment combined with boric acid, and found that not only did it act efficiently upon the thickened masses, but even in those cases seen in the later stages of trachoma where trachomatous pannus had supervened, I found that the pannus was appreciably thinned, and the inflammatory reaction diminished. I believe the honor of having first instituted this type of treatment belongs to me, and certainly it has proved in my hands a most efficacious method. I agree with Fuchs in his conclusions, after trying all of the methods, both irritative and conservative in their tendencies, that all harsh surgical methods should be eschewed in trachoma during its early and transitional stages, and should be reserved for the correction of the numerous disastrous sequelæ, such as pannus, entropion and ectropion, distichiasis and trichiasis, lachrymal obstruction, madarosis, tylosis, corneal opacities, etc., which have all been heretofore noted as results of the typical siege of trachoma or granular lids.

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PROFESSOR KOCHER ON MODERN PROJECTILES IN WARFARE

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"In the time of peace," it has been said, "prepare for war." This necessary evil must sooner or later come to all countries, judging from the past, its evil consequences are sufficient when happy homes are robbed of fathers, husbands and sons, but when the projectiles are of such a nature as to produce great destruction of tissue, owing to shape and velocity, it becomes all true philanthropists to assist the Red Cross, which will figure so conspicuously in future Italian wars, to bring about such a change in projectiles as will cause the least destruction of tissue compatible with "disabling the enemy and causing him to retire from the field." During the riot in this city in 1893, I had the opportunity of seeing the great amount of mutilation and serious wounds produced by the modern firearms in the hands of our military company—those not instantly killed are so mutilated by large projectiles that permanent disability for manual labor is the rule, while the chances for infection from the wounds are very much increased. It becomes all civilized nations, from a humanitarian point of view, to repudiate the idea expressed by Shakespeare in "Henry IV," that "the arms are fair when the intent for bearing them is just," and so construct all projectiles as to disable with a minimum amount of mutilation—rendering survivors more useful and comfortable. With this end in view I have submitted an abbreviated report of a lecture to which I listened at one of the general meetings of the International Medical Congress in Rome, Italy, in April of this year, delivered by Kocher, of Berne, Switzerland, than whom no one is more competent to speak, as his researches in this line have been very exhaustive, and his investigations respected and indorsed by the civilized world.

This subject may embrace all of us in its scope, and the military and naval officers and authorities should seriously consider this lecture on the subject of "the improvement of projectiles from a humanitarian point of view." Kocher said:

"The reason why ancient Rome, whose hospitality we are now enjoying, acquired the immortal glory which it maintained through the classic ages till our own times, is to be found in the warlike character of its first inhabitants, in the *Virtus Civis Romani*. Notwithstanding the great progress which has taken place in the field of intellectual activity, it is still the case that those nations become arbiters of the world who are always ready to sacrifice their life and all they possess for liberty, truth and justice.

"Military service is a duty which extends to all the citizens and therefore all take an interest in the necessary and inevitable consequences of war. Women and sanitary associations vie with each other to diminish the sufferings of the victims, as also the Red Cross, whose representatives showed us two years ago in this same city the splendid results obtained by them.

"But all this is of no use (I speak simply from a surgical point of view) if modern firearms bring such devastation in the contending armies, while they should only put *hors de combat*—if they mutilate instead of simply wounding. Twenty-five years have

not passed since it was universally recognized that perfected firearms cause enormous damage in the human body. This general opinion is confirmed by the recent communications of Démosthène, Chauvel, Nimier, Bogdanik, Sklissowsky, Morosow and Trauber, by the observation of Kabars and finally by the interesting experiments executed by order of the Prussian Minister of War, by Dr Schjerming all of which show the terrible effects of modern bullets. After this must we await further improvements or must we resignedly accept the conclusions of Surgeon-General Dr Von Coler and of Dr Schjerming, namely, that with the new rifle life must absolutely be lost?

"The object of war between civilized nations is not, as among savage people to kill the greatest number of combatants, but to disable the enemy and to oblige him to abandon the field. Nevertheless there are many unexpected deaths caused by injuries to vital organs. But the modern guns surpass greatly this scope, because they tear or destroy the tissues of the body, exposing the victim to the risk of losing his life even through non-vital injuries, and either oppose or prevent entirely the reestablishment of the normal functions. Guns have become real explodent firearms, which are prohibited by international agreement.

"In order to eliminate the damages produced by these arms it is necessary to know the effect of the explosive projectiles, but unfortunately on this matter the opinions of the many authors are too different.

"I will demonstrate, considered by twenty five years of experience, in what really consists the explosion and by what it is caused, this will be the subject of my lecture. The action of the bullet is different as the object which it strikes varies. We shall therefore distinguish three separate categories in the human body. Elastic tissues, tissues rich in liquid substances, and rigid tissues.

"In elastic tissues the bullet does not explode, at least not immediately, even if fired out of the most modern rifle. Large lacerations of the skin are due to deeper explosions, such as those which take place in the vicinity of the bone, and without these external lacerations are not to be found. In some elastic tissues we can only observe the point of entrance and exit of a bullet whose initial velocity is of 600 to 750 meters, we can not trace any hole at all as the tissues after having been forced aside, resume their former position.

"The effect of an explosion may be observed with far greater facility in tissues which contain an abundance of liquid substances, and is naturally more terrible in those tissues which more abound in them. Brisch was the first who had recourse to hydrostatic pressure in order to demonstrate the effect of an explosion in the cranium, Garfeuil, Heppner and Kuster applied the same principle to epilepsy, and I have been able to show by means of numerous experiments the general importance of liquids in the tissues. The action of hydrostatic pressure may be demonstrated on tin boxes containing substances more or less rich in water. The effect of the explosion differs greatly if the box contains fresh horse flesh instead of dry, damp cotton or saw dust instead of dry.

"Here are some elastic laminae that present a very small hole when the velocity of the bullet is small, while enormous rents are made by it when the

velocity is at its maximum. These violent effects are caused both by leaden and by steel bullets, it is therefore impossible that they should be produced by the deformation of the bullet, as it is proved that steel does not undergo any alteration of shape. In any case it is important to observe how the explosion is influenced by the different degrees of deformation if the area which is struck becomes larger.

"Demonstration of the effects of exploding bullets on recent epiphysees, on the glandular and visceral organs and on the muscles. The degree of intensity of the explosion gradually augments with the increase of velocity, while we can only talk of hydraulic pressure in the case of maximum velocity and on parts like the cranium.

"The effect is seen in its full intensity only if the distance is great enough, but also in open tin boxes, where the water is not hermetically closed as in a hydraulic pump a laceration takes place. It is therefore better to abandon the name of hydraulic or hydrostatic pressure and to use that of hydrodynamic laceration.

"The speaker afterwards shows the effect of explosions on flexible substances, such as soap or glass, in which we may with certainty establish, by means of comparative measures of weight and volume, whether we have to deal with a simple shock or with a missile that produces at its maximum velocity the maximum degree of dilatation.

"Also here we find an augmentation in the explosive effect even though there be no rotation or deformation and we must recognize that the intensity of the explosion does not depend from these factors.

"The real nature of an explosion can only be understood if its effects are studied on a solid body. To this end we can fire a bullet at a slab of stone, and we will ascertain that the shock transmits itself with great violence a great distance. The difference between a simple crack and a real laceration may very well be distinguished in a glass slab if the velocity of the missile is not at its maximum, in which case a number of cracks are produced instead of a neat small hole. The best manner of observing the perfect analogy of the effect of the dry explosion with those of hydrodynamic pressure on a tin box is to fill this box with small shot or pieces of stone.

"Numerous illustrations demonstrate the analogy of explosion in dry and fresh bones, which is then the essence of the explosion? Kocher has been able to demonstrate by determining the velocity of the bullet directed to different objects and also on the human body, that for maximum velocities a greater amount of active energy is lost than for the small ones. This augmentation in the loss of active energy is caused by the velocity being too great to pierce the target and by remaining longer imbedded in it produces an unequal shock. By means of measures it is possible to ascertain that the loss of velocity and active energy is greater in bullets of heavy caliber, and consequently the lacerations will be greater.

"Conclusions to improve the projectiles from an humanitarian point of view.

"1 The cause of the laceration can not be avoided because it depends from the augmentation of velocity.

"2 It is necessary then to modify the importance of the laceration which happens

"3 By diminishing the area struck
 "a, reducing the caliber of the projectile to five to six centimeters,
 "b, preventing the deformation by giving it more compactness especially in the point,
 "c, by making the anterior extremity sharper so as to increase its strength of penetration,
 "d, by augmenting the rotation so as to prevent the oblique penetration

"Ulterior experiences will demonstrate if to solve the problem from an humanitarian point of view it will be necessary to use again very small copper projectiles or projectiles with steel coating and point. The results already obtained by the introduction of the steel coating advise us to follow on the same track. 1, even after the worse results there are no more muscle and lung lacerations, 2, the fracture of bones is totally limited in extension and intensity, so short the hole of exit is, according to the experience of Collier and Schjerming, reduced for 200 meters distance from 2 to 3 centimeters while before it varied from 7 to 13. One of the greatest improvements from a medical point of view is the reduction of the hole, as the healing of the wound is then materially facilitated and dangerous complications are rendered less frequent. But the extraction of large fragments of bone, and injuries to vital organs such as the heart, the brain and the lungs will always make numerous victims, and also in the next war hundreds of soldiers will end their life on the field of battle.

"But the great mass of our youth who courageously march into the grim jaws of war will be protected by a new Pallas, armed with the shield on which shines the Red Cross."

SOCIETY PROCEEDINGS

The Canadian Medical Association

The annual meeting of the Dominion Medical Association was convened in St. John, N. B., August 22 and 23, 1894. The meeting was well attended by medical men from nearly every province, the larger number coming from the Maritime Provinces. The Association had not met in St. John before for twenty years. Fraternal delegates were introduced. Dr. J. M. Jonah, of the Maine Medical Association, Dr. Bulkley, of the American Medical Association.

The chair was occupied in an able manner by Dr. T. S. Harrison, of Selkirk.

The subject of

THE TREATMENT OF EPILEPSY

was first taken up, the paper being presented by Dr. Hattie, of Halifax. He gave a description of the various changes that have been said to take place in the cerebral cells during fits of different kinds, and pointed out that the causation might be due to the influence of some irritant acting upon the cerebral cells, which put them in a state of excitability, or which, by being produced in the encephalon, and not eliminated quickly enough, by a nerve explosion was gotten rid of—that the convulsions were due to an abnormal functioning of the cells in some part of the nerve centers, the location determining the character of the seizure. This poison might be generated in the system. If this were the case, the rational treatment would be the eliminative and antiseptic one. This he had tried, by giving in addition to the bromid of potassium, beta naphthol. The results were very gratifying, the number of fits per patient having been materially reduced. Dr. Muir then gave the history of

A CASE OF TUBERCULOSIS OF THE ARM, BETWEEN THE ELBOW AND THE WRIST,

which had been in existence for some fourteen years, with all the typical symptoms (the tubercle bacillus being present), which, after being scraped, was infected with erysipelas. Subsequently complete cure took place.

Dr. SHEPHERD reported a case of cancer cured in this way.

Dr. CAMERON said he had tried to inoculate some cases of malignant growths with the germ of erysipelas, but had failed to get it to work, probably because the sarcomatous and carcinomatous germs overcame the invaders.

SIR JAMES GRANT said that he had seen whooping cough very materially relieved in children who had been vaccinated. He had tried vaccination in certain cases of psoriasis he had had, with extremely gratifying results. He believed there was much room for study along this line.

The President of the Association, Dr. HARRISON, took as the subject of his address his experience in medical matters during the last half-century. That long ago he settled in the early wilds of Ontario, near Lake Erie. Those were the days of ague, bilious remittent fever, murrain in cattle, and other diseases which now, since the country is cleared up, have become almost extinct. He gave a graphic clinical history of these various diseases and the different forms of treatment employed, both before the doctor arrived in the settlement and after, by those who sought to charm the disease away and those who sought to treat it according to the tenets of Thompson. Whisky, in those days, was considered by the people to be the sovereign remedy. Some doctors bled, blistered and salivated. The President told of difficult experiences he had had alone far removed from medical help in various kinds of operations, major and minor. The latter part of the paper was an advocacy of the establishment of uniform examinations for all who wished to practice in the Dominion, and that the standard should be that of the highest provincial standard. The President was accorded a hearty vote of thanks for his address, and a committee was appointed to consider the matter of reciprocity discussed in his address.

The subject of

APPENDICITIS

was discussed by Dr. BELL, of Montreal. He reported forty-eight cases, forty of them operated on with recovery, five not operated on, and only three deaths altogether. He classified his cases into the gangrenous, the perforative, and those bound in with adhesions. These cases should be watched, he maintained, by a surgeon from the first, as little could be done for its relief medicinally. He advocated surgical interference in nearly all cases.

Dr. HINGSTON thought the operation was performed unnecessarily. No young man should attempt to enter the abdominal cavity without first consulting one or two others. He had prevented the operation twenty-five or thirty times, and only regretted this step in one case. He was strongly in favor of conservatism.

Dr. GRANT, Ottawa, spoke of two cases he had had which appeared to be fit ones for operation, but one was in a gouty subject, the other in a rheumatic. He did not operate and they recovered. It was very difficult to know what to do in these cases. He did not believe appendicitis was due to the presence of foreign bodies in the organ.

Dr. SHEPHERD pointed out that the surgeons got the worst cases, so it was difficult to say just what the number of cases was which were operated upon as compared with those that were wholly treated medicinally. His idea was to operate after the acute attack had subsided in the interval. He attributed the tender point of McBurney, not to the appendix, but to the inflamed condition of the mesenteric glands.

for the appendix might be found on the right side in the pelvis, or up under the liver

DR STRANGE was in favor of non interference until there was the presence of a tumor and pus. He had refrained from operating during the acute stage and had not regretted it.

MR CAMERON, of Toronto said he followed Mr Treves in this matter, to wait until the pus formed, open and drain. He considered it unfortunate that the experience of a hospital surgeon of skill, should determine the matter one way or the other. In a possible forty eight cases he had had in which he had followed out the above treatment he had had probably, Dr Bell's figures reversed. The interval he believed to be the time to operate.

In replying to the discussion on his paper, Dr Bell made a strong plea in favor of his statement, "one should always operate." It was generally agreed that no one knows when to operate. If the patient were left at any moment, perforation might take place. However, in the forty cases he had operated on, thirty were perforated and abscess was present at the time of operation. In three the appendix was wholly gangrenous. And in such, he said, one could not wait for the tumor formation or the abscess, because there was none. In two the appendix was bound down, in three the appendix was not perforated, but gave rise to urgent symptoms, yet there was no abscess found. He used to follow the waiting treatment but found it unsatisfactory. The mortality was much greater than that of his eleven months of the new plan. The greatest mortality statistics for the operation only amounted to from 2 to 3 per cent. The operation, as a rule, was not difficult. He considered the plan of waiting for pus not the best surgery. The very mild cases where the symptoms passed off in say twelve hours, he would not interfere with. They were probably only cases of cecitis.

DR MORRISON, of St John, read a paper entitled

EYE STRAIN HEADACHES

It had been alleged that 90 per cent of all headaches was due to eye strain. This he believed to be true. Many of such were attributed to other causes, as biliousness "womb trouble," nervousness, masturbation, over work, etc. when the real cause was overlooked—an over-worked condition of the muscle of accommodation—ciliary muscle. This condition of the delicate muscle was brought about by attempts to correct varying degrees of astigmatism. No cornea hardly had perfect curvature in every direction, and it was these slight degrees of curvature, often overlooked even by the specialist that lay back of these headaches. The use of cylindrical glasses with low dioptric power, always relieved the muscle and consequently the headaches. Constitutional treatment was also advocated, and the avoidance of those conditions of life that tended to increase the trouble.

The subject of

DISEASES OF THE OVARIES AND TUBES

was by DR SMITH, of Montreal. In gonorrheal salpingitis the clinical history of which he went into the only safe treatment was extirpation. This was also the course to pursue in the tubercular form, if the general constitution were not too much infected with the poison. He advised medicinal treatment for the functional disorders of the appendages. The paper was illustrated by a number of interesting cases, and the presentation of tubes and ovaries that he had removed.

THE USE AND ABUSE OF THE VARIOUS CAUTERY AGENTS IN THE TREATMENT OF NASAL AFFECTIONS

was treated by DR. E. A. KIRKPATRICK, of Halifax. He referred first to the delicacy and importance of the nasal mucous membrane, and said that too often it was the subject of

too harsh treatment. Caustics were used, perhaps, more in hypertrophic rhinitis than for anything else, and often too severely. Of the caustics he used chromic acid, trichloroacetic acid, and the electro cautery were the principal. The chromic acid he used in anterior applications, the cautery for the posterior applications. By the injudicious use of caustics he had seen the mucous membrane destroyed. And in some cases he had seen very serious sequelae follow in connection with the ear, such as loss of hearing and mastoid disease.

The address in medicine was delivered by DR. BAYARD, of St John, N. B., subject

THE INFLUENCE OF THE MIND ON THE BODY

This was, he claimed, a subject of growing importance in this rushing age. Most authorities were agreed that surgery and medicine were rapidly advancing. It was also agreed that nervous diseases were on the increase, particularly insanity and neurasthenia. This was largely due to the energy, competition, worry, compulsory education, sensational novels, newspapers, speculation and unrest, which characterized the last part of this century. Another cause was the migration from the country into the town, where the strife for existence was greater and sanitary surroundings bad. Relief from this condition of affairs was largely through the instrumentality of educational reform and the employment of preventive measures generally.

As an outcome of one of the points referred to in the address, at the suggestion of Dr Hingston, Dr Bayard moved, seconded by Dr Hingston, "that the system of education generally pursued in the Dominion of Canada draws too largely upon the brain tissue of children and materially injures the mental and bodily health."

DRS CAMERON, of Toronto and Powell, of Ottawa thought the terms of the resolution were too sweeping, that there was no specific statement as to what department of the school system was at fault, nor to what portion of the Dominion it more especially applied. Our young people, Dr Cameron thought, were not suffering the older people neither, from too much education. The educational system had been the subject of the best thought of our best men and he considered the motion too condemnatory. A resolution was then passed that the matter be referred to a committee, consisting of Dr Powell, Dr Hingston, Dr Graham and Bayard.

DOMINION REGISTRATION

The committee appointed to report on the President's address, reported on the matter of inter provincial registration. It was adopted.

DR DANIEL moved, seconded by Dr Powell, "that a committee be appointed in which each of the Provinces shall be represented, to draw up a form of medical act, which, after being adopted by this Association, shall be presented to each Provincial Legislature to be by them passed into law and that the committee that brought in the report be asked to name such committee."

DR BULLER moved, seconded by Dr Laphorn Smith, that committee be appointed, with power to add to their number to consider the best means of obtaining a uniform standard of medical education for the Dominion of Canada, and that said committee report at the next meeting of the Association. This carried.

The discussion over the above question was long and animated, and taken part in by several of the men from the different provinces represented at the Association.

DR J. E. GRAHAM presented a paper on

SOME FORMS OF FUNCTIONAL DERANGEMENTS OF THE LIVER.

The paper dwelt on the great importance of maintaining the integrity of the hepatic cell. It acted as a guard against

the invasion of certain forms of poisons into the general circulation, such as arsenic and phosphorous among the mineral kingdom, the poison of decaying meats and old cheese, in the organic, absorption of poisons generated in a distended stomach, the specific toxins of typhoid and such diseases, and the absorption of poisons from the intestinal tract, say when constipation was present. These various poisons tended to hurt the cell, and passing into the general circulation had their effect on the nervous and other systems of the body. In the ordinary cases of biliousness the symptoms were caused in this way. The glucogenic and urea producing functions of the liver were also discussed and the results pointed out, of their abeyance, through the damage done to the hepatic cell. As to treatment, Dr Graham pointed out the absolute necessity of finding out the exact cause of the disturbance before rational treatment could be employed. He recommended restriction and regulation of the diet, suitable exercise to assist the circulation, proper attention to bathing, drinking of mineral water to assist metabolism, where the urea was deficient, the necessity of free purgation, and lastly the various specific remedies recommended for this condition.

Dr. HINGSTON reported

FOUR CASES OF OPERATIONS ON THE BRAIN

The first two were for epilepsy. The first without the results hoped for. The second was operated on for cephalic pain, located in one spot. It had been incessant and severe for a year. The Doctor trephined and found a hydatid pressing on the brain, pediculated, which he removed. The patient made a good recovery. The next case was that of a young man, whom the Doctor presented, who had suffered for twenty years, as the result of a fall and injury to the right side of the brain. He was the subject of paralysis of the left arm, which was drawn up and flexed, the fingers also being flexed in their terminal phalanges and extended in the first. The orbicularis and zygomatic muscles and the others on the left side were spasmodic and overdeveloped, the pupil was contracted the vision and hearing on that side impaired. On operating, a thickened portion of bone was found impinging on the brain tissue, surrounded by a cartilaginous material which nature had thrown about it. There was no bleeding upon its withdrawal. The expression of the face at once became relaxed, and the patient seemed almost complete in his facial appearance. The arm and improved. Dr Hingston recommended the use of a large trephine, two inches in diameter, for these operations. Dr SMITH, of Montreal, gave the history of a case of removal of the entire upper limb for a chondro sarcoma involving the shoulder joint, also of the removal of a large enchondroma of the pelvis. The first operation had not been done often, his being the first that been done in Canada. Drs Hingston, Cameron and Steeves discussed the paper. Dr BUTLER read a paper on "The Present Status of Asthenopia."

Dr INCHES, of St John, N B, read a paper entitled

THE PREVENTION OF TUBERCULOSIS,

in which he advocated the necessity of increased activity on the part of the profession, the public and the government in dealing with this dangerous disease. The patients themselves needed much instruction in regard to the destruction of the sputa, so as to lessen the danger of infecting others in the house. Even in well kept consumptive hospitals there was a little danger. He dealt with the difficulties connected with notification and registration and isolation. He had found it very difficult, even among his wealthy patients, to secure isolation and fresh air, and it was infinitely more difficult to secure such among the poorer classes. Special sanatoria, he maintained, should be provided, and in every instance where the patient was not properly looked after at home, he should be sent to such places. Until such a time for there are very few as yet, those cases should be reported when preventive measures were not carried out thoroughly at home as recommended by the patient's physician.

Dr L DUNCAN BULKLEY, of New York, gave a paper on

THE TREATMENT OF SKIN DISEASES

More success would come to the general practitioner in the treatment of the skin if more attention was paid to each individual case. He advised careful inquiry into every detail of the patient's system and habits. The history of the eruption, careful inquiry as to former eruptions, family tendencies as to presence of asthma, rheumatism etc all should be made a note of. If medical men knew eczema,

acne, syphilis well, they would be able to treat the great majority of their cases satisfactorily. As to eczema, too much was often done, it was over treated often. More and more he had grown to know that much depended on constitutional treatment in all these skin affections. The correction of some fault in diet or habit in life was sufficient to effect relief. The Doctor pointed out some of the principal points in the management of acne, syphilis, psoriasis and urticaria.

THURSDAY EVENING

The report of the committee appointed at the last Association to consider the matter of the establishment of a pharmacopœia was received and adopted. On motion of Dr Starr, seconded by Dr Macdonald, it was moved, "That the same committee be requested to correspond with the different medical and pharmaceutical associations with regard to the advisability of publishing a pharmacopœia, taking the British Pharmacopœia as a standard." Carried.

THE PREVENTION OF CONSUMPTION,

was the subject of a paper by Dr J F MACDONALD, Nova Scotia. He advocated the bringing the matter of the contagiousness of the disease before the people by means of the secular press, by the establishment of philanthropic societies for the discussion of the matter, and the adoption of practical measures for the treatment of the cases. He advised the system of registration, a careful system of disinfection, government inspection of infected places, the establishment of sanatoria, and the enactment of laws to prevent the infected from spreading the infection.

Dr H D HAMILTON read a paper on "The Adhesions of the Soft Palate and Their Treatment."

Dr J T STEEVES, of St John's Lunatic Hospital, read a paper entitled "A Medico-Legal Romance." It was discussed by Drs Muir, Macdonald, Morrison, Christie, Hattie and Travers.

Dr FENWICK then presented a paper on "Hysteropexy."

The Association then adjourned to meet next year in Kingston, Ontario—*Dominion Medical Monthly*

NECROLOGY

HERBERT GOUDE, M D, who for fifteen years had been resident medical officer at the Highgate Smallpox Hospital has recently died in London, in his forty-eighth year. He was a man of unquestioned fitness for his position as a successor of Dr Marson at the Highgate institution, and his loss is spoken of with universal regret. He was able to continue the observation and testimony of his predecessor there and, as recently as in May of last year, was enabled to write regarding the unbroken record of fifty eight years that no nurse or servant had contracted variola even in modified form, with the single exception of a gardener who evaded vaccination, and this, too, despite the fact the nurses have actually and accidentally inoculated themselves with variolous matter. This he may well term the crucial test of vaccinal protection, and the unanswerable argument in favor of the operation. Dr Goude was the author of full history of his hospital, from the time of its foundation in 1746 at Battle Bridge, King's Cross, up to the present time. As the *Lancet* says "He sought to enforce the great lesson of the value of vaccination that is still unlearned by thousands, and history will not fail to own its obligations to him and his fellow laborers."

HANBURY SMITH, M D, died at Brooklyn, N Y, on September 12, aged 84 years. He was a native of Staffordshire, England, and a graduate in medicine at a London school in 1831. He practiced for several years in Stockholm, Sweden. He came to this country in 1847, and resided in Olden about ten years. In 1859 he took up his residence in New York city, and became a pioneer in the introduction of production of mineral waters for the use of the profession and the public. He is survived by one son, Mr Bernie Smith at whose home he died as above stated.

HENRY HOFFMAN-DONNER, M D, the eminent physician, humorous writer, and poet, died at Frankfort on the Main, September 20, aged 83 years. His chief humorous work was "Strawelpeter," which ran through 150 editions and translated into many languages.—Dr Rush, of Macon, Georgia, September 14.—R P Watson, M D, September

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SATURDAY, SEPTEMBER 29, 1894

ON ARTIFICIAL TEETH

A writer in *Les Nouveaux Remedies* for Sept 8, 1894, says that "the teeth of the Americans are without doubt the worst in the world." "Also," says this most interesting person, "this eminently practical people have long since renounced the employment of the inferior products furnished by nature, for their substitute, artificial teeth." The writer then produces some statistics to show that some 8,000,000 false teeth have been sold in the last year. He also cites the statistics of M MAX DE NANSOUTY, who has made a calculation to show the number of pounds of gold used in filling teeth in the United States. This amount, he asserts, is not less than 800 kilograms which represents about 2,500,000 francs. All this precious metal we are told, "is buried with the Yankees when they die," and on this assertion this MARTINUS SCRIBLERUS splutters, "supposing that this continues during some hundreds of years, the un pitying statistician estimates that in three centuries, the cemeteries of the United States will receive a value of 750,000,000 francs in fine gold, equal to the money value of the gold which actually circulates in the country. There is a new California in prospect for the gold seekers of the twentieth century!"

We did not need this sneering ebullition to let us know the great advance America has made in civilization and in the humanities. The French Government sent one of the best native dentists in Paris to attend the International Medical Congress of 1887 and to report on the dental schools and the state of dentistry in the United States. His report was printed in an elaborate volume entitled "*L'Art Dentaire de l'Etat Unis*." In that official report, which

is complimentary to the United States in the highest degree, the writer regrets that France is so far behind the United States in the dental art. The superiority of American dentists has been acknowledged by the world for more than two decades, and an American dentist (EVANS) in Paris received more decorations of honor than any professional man of his time, whether in his particular field or out of it.

Nor is this superiority the result of the vast number of cases of bad teeth, as our contemporaries would have us believe. It is because the Americans have passed beyond the dentistry of the Middle Ages, and realize that sound teeth are conducive to longevity. The filling of teeth is neither new nor peculiar to Americans, and it is doubtless true that the ancients understood the care of the teeth much better than the modern French. The third ÆSCULAPIUS is usually given credit for being the first to extract teeth, but the Egyptians understood their preservation well, and GALEN introduced into the carious tooth a pellet of wax for the toothache, and as a permanent cure he first cleared the cavity with acid and then filled it with powdered pyrethrum with resin or wax as an outer covering. Indeed, the ancient Romans took great care of their teeth, according to PLINY, washing and rubbing them repeatedly, and as we learn from HORACE (Sat I) when they lost them they had them replaced with ivory teeth, and when loose they bound them with a thread of gold. Tripoli powder ("red soda") was much used as a dentifrice,¹ and the ancient surgeons, subsequent to CELSUS, punctured alveolar abscesses with the red hot iron. The putative father of French surgery, GUY DE CHAULIAC, (its real father was HENRI DE MONDEVILLE) made a serious complaint that the pulling of teeth had been abandoned to barbers and dentists, and he held that the operation was sufficiently important to be attended to by physicians, but even GUY recommended the filling of cavities with aromatics and resinous substances. SPRENGEL asserts that JOHN ARCULANUS, the Veronese, (quoted by RHazes) was the first to use gold leaf for filling teeth, but judging from the remarks of our contemporary, his recommendation fell on stony ground, when it fell in Gaul.

In the matter of the construction of artificial teeth, ivory was alone used, down to the time of ANTOINE NUCK, (he of the Canal) who insisted that the only proper ivory was from the tusk of the hippopotamus. DUBOIS DE CHAMANT (1777) was probably one of the earliest to use porcelain teeth. But, like every solid advance in the other branches of medicine, it has been a slow progress, and the present advanced position has been reached by regular steps. The country of our contemporary has done little or nothing in the last century toward the advancement of this branch of surgery.

¹ One can scarcely resist a reference to Catullus "Upon Egnatius and To the frequenters of a certain Tavern wherein the dentifrice used by the Celtiberians is held up to ridicule."

heart disease, subacute bacterial endocarditis and congenital heart disease. Contrary to our apprehensions, the biopsies were uncomplicated and no hemorrhages were encountered. The material was fixed in formol saline and stained routinely with hematoxyline and cosin, reticulum stain by the silver impregnation technic of Gomori, and Mallory's picric stain for connective tissue. Iron pigment was stained by the Prussian blue method.

The clinical severity was graded by an arbitrary method of labelling each predominant symptom by a number of pluses. The biopsy was performed within three days of gradation.

Table I shows the five criteria selected for judging the clinical severity of congestive heart failure and their grading:—

TABLE I.

Showing criteria for judging severity of congestive heart failure.

Symptom.	GRADES OF SEVERITY.			
	1+	2+	3+	4+
1. General appearance of patient.	Apparently well	Slightly ill	Moderately ill	Very ill
2. Dyspnoea ...	On moderate exertion	On walking	At rest	Orthopnoea
3. Veins in neck with patient at 45° inclination	Just seen	Up to middle of neck	Up to angle of jaw	Full
4. Edema ...	Feet or face	Feet and legs	Feet, legs and abdomen	Generalised or with ascites
5. Liver enlargement ...	Up to 1"	Up to 2"	Up to 3"	More than 3"

Simultaneously, a composite liver function study was performed on every patient. This included the total proteins, albumin and globulin, (King, 1946), colloidal gold flocculation test (MacLagan, 1944), prothrombin time determination (Iyengar *et al.*, 1940), bromsulphalein retention (Rosenthal and White, 1925), urine urobilinogen (Wallace and Diamond, 1925) and serum bilirubin (Malloy and Evelyn, 1934).

A 24-hour sample of urine was subjected to a paper partition chromatographic analysis using the double dimensional technic of Williams and Kirby (1948) with phenol and butanol acetic acid water as the solvents, 0.25 c.c. of urine was used.

The extent of centrilobular hepatic necrosis was graded as follows:

Grade A.—Hepatocellular changes confined to the region immediately adjacent to the central vein (Plate 1, fig. 1).

Grade B.—Liver cell necrosis confined to the central 1/3rd of the lobule (Plate 1, fig. 2).

Grade C.—Liver cell necrosis extending beyond the central 1/3rd of the lobule (Plate 1, fig. 3).

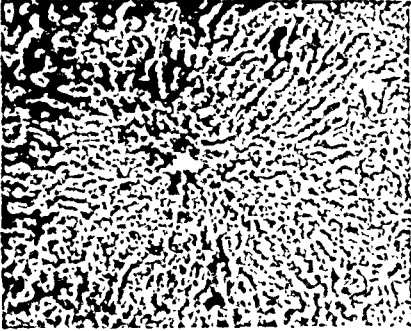


FIG. 1. Grade A centrilobular hepatic necrosis with changes confined to the region immediately adjacent to the central vein; $\times 150$.



FIG. 2. Grade B centrilobular hepatic necrosis with changes extending to about central 1/3rd of the lobule; $\times 150$.

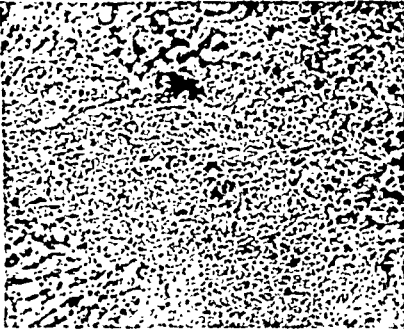


FIG. 3. Grade C centrilobular hepatic necrosis with changes involving major part of the lobule; areas of frank hæmorrhage can be seen; $\times 150$.

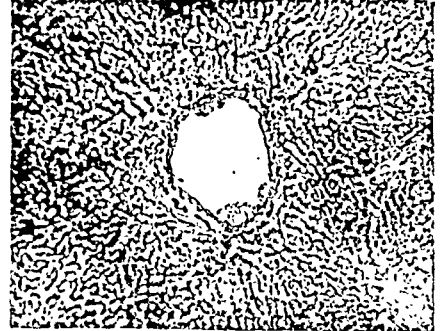


FIG. 4. Dilated central vein; $\times 150$.



FIG. 5. Central vein with phlebosclerosis of its wall; $\times 120$.

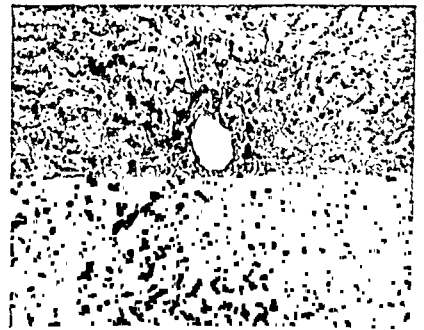


FIG. 6. Dilated central vein and dilated sinusoids with narrowed and disorganized liver cell columns; at places liver cell have completely disappeared; $\times 150$.

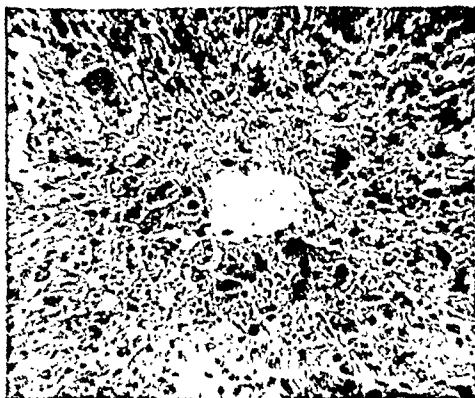


FIG. 7. Necrosed areas showing cellular invasion and capillarization; $\times 344$.

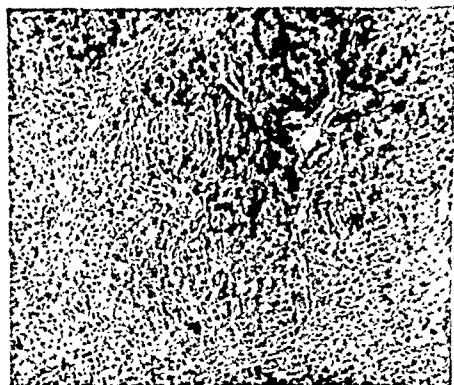


FIG. 8. Collagenous and fibroblastic transformation of necrosed areas. Slender connective tissue septa are seen extending to neighbouring central ones; $\times 120$.

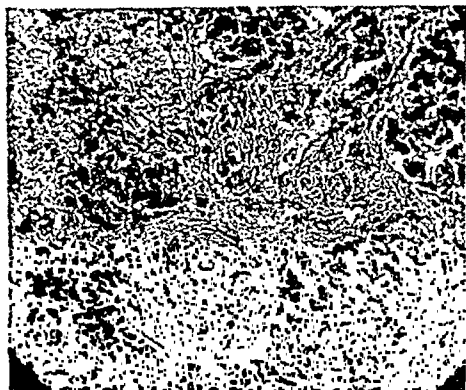


FIG. 9. Fibrous tissue bands showing extensive neoangiogenesis which brings about short circuiting of the blood channels; $\times 150$.

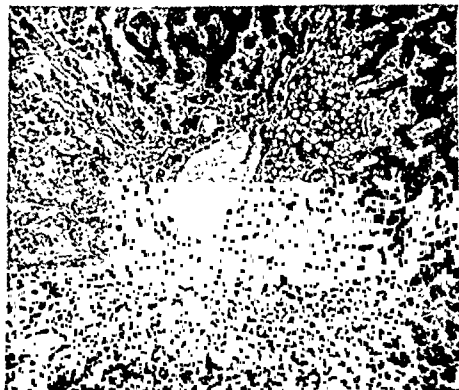


FIG. 10. Dilated central vein with marked fatty changes in the central portion of the lobule; $\times 150$.

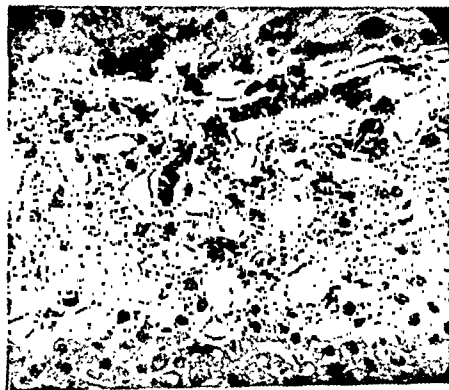


FIG. 11. Dilated spaces of Disse; $\times 645$.

TABLE II.

Showing the relationship of the clinical grades of congestive failure with the severity of central necrosis.

Clinical grades.	GRADES OF CENTRAL NECROSIS.		
	A	B	C
+	3	2	...
++	2	3	...
+++	1	3	5
++++	...	4	6

The pathological severity of the congestive failure was also related to the duration of the disease, the longer duration of the disease being related to greater severity of central necrosis. None of the patients with type C change had been in failure for less than 3 months. Only 2 of 12 patients with type B change and no patient with type A change had been in failure for more than 2 months. Central necrosis was seen in all etiological types of congestive heart failure but the severer grades were more common in mitral stenosis. Mitral stenosis was also the most common cause of fibrotic changes of any consequence, 4 out of 5 cases of cardiac fibrosis, being caused by it. All these patients had repeated attacks and remissions of congestive failure, the shortest duration being two and a half years.

Liver function studies.—Of the 61 cases studied, 10 had hypoproteinemia. Disturbance of the albumin and globulin values was, however, in a greater number of cases, e.g. hypoalbuminemia and hyperglobulinemia in 40 cases. Thymol turbidity values were above normal in 6 cases, and in 13 cases the thymol flocculation and colloidal gold flocculation tests gave positive results. Cephalin cholesterol flocculation was positive in 35 cases. Bromsulphalein test was positive in 29 cases. Urine urobilinogen values were inconclusive as the excretion showed fair degree of variation from day to day in a large percentage of cases. Of the 61 patients, 25 patients had serum bilirubin higher than 1 mg./100 c.c. However, an appreciable degree of jaundice with serum bilirubin greater than 3 mg./100 c.c. was present only in 3 cases. It was noted that the abnormalities of the function tests were not closely related to the clinical severity though generally speaking the patients in advanced circulatory failure had a greater aberration of the function tests. Graph 1 represents the pattern of hepatic function tests in a composition function chart.

A correlation of the grade of pathological change in the liver with the abnormality of the liver function tests was only possible in the 29 cases whose specimens were available for histological study. Hypoproteinemia was not related to the degree of liver damage. Hypo-albuminemia and hyperglobulinemia was present only in grades B and C. Amongst the flocculation tests, only the cephalin cholesterol test showed a graded positivity, e.g. being positive in 9 out of 15 cases in grade B and 7 out of 11 cases in grade C. Bromsulphalein test was positive to a significant degree (above 10 per cent) in only grade C, being positive (10 to 40 per cent retention) in 9 out of 11 cases. The mean serum bilirubin values

tended to be higher in patients with severer degrees of hepatic damage. In two cases of grade C, it was higher than 3 mg./100 c.c. and in 4 out of 12 cases of grade B, it was higher than 1 mg./100 c.c. It was uniformly below 1 mg. in all cases of grade A.

GRAPH 1.

[illegible]

Composite liver function study chart showing pattern of hepatic function test results.

Study of amino-acid metabolism by paper-partition chromatography.—Generally speaking, in the clinical 3+ and 4+ grade cases, a tendency to amino-aciduria was seen to be present. Contrasted with a normal pattern in which usually only 3 amino-acids are present (glycine, alanine and histidine), an average case of advanced congestive failure showed the presence of as many as 16 amino acids (Graph 2). In some cases, the glycine, ornithine and histidine spots tended to be the strongest in intensity of colour. This amino-aciduria also tended to diminish with the clinical recovery of the patients.

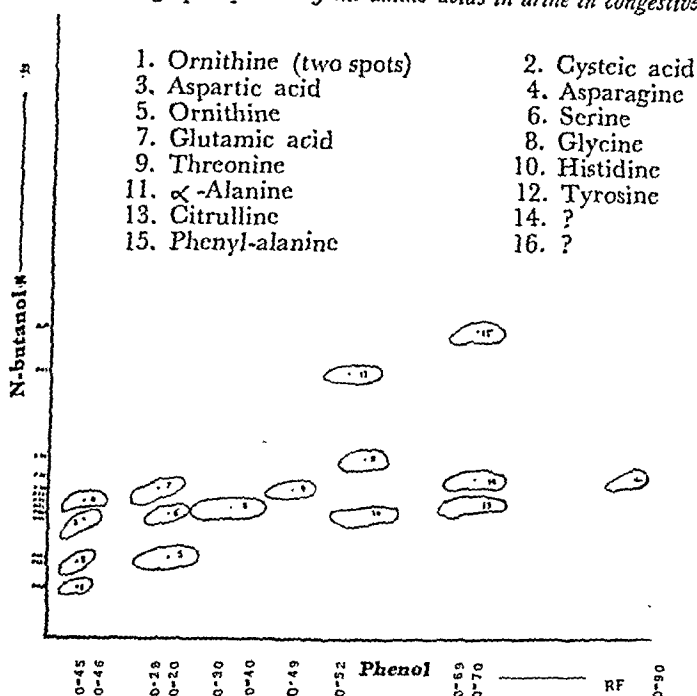
DISCUSSION.

A noteworthy observation in the above study is the almost invariable finding of central necrosis. Several explanations have been offered for this change. The peculiarities of the blood supply have, in a large measure, been incriminated. Liver is the only organ in the body which is supplied predominantly by venous blood. Less than one third of the total blood supply of the liver is derived from hepatic artery (Macleod and Pearce, 1914). Moreover, the branches of the hepatic artery in the interlobular septa average 8 microns in diameter compared with the 9 to 10 microns in diameter of the portal vein. The liver cells at the centre of the lobule receive blood at a lower oxygen tension than at the periphery (Blalock and Mason, 1936). Also, by the time the blood reaches the central vein from the portal system, it is deprived of much of its meager share of oxygen content. Hence, the centri-zonal cells are on a precarious oxygen supply and any fall in its level even for a short time is apt to produce degenerative changes.

Liver Damage in Congestive Heart Failure.

GRAPH 2.

Double dimensional chromatographic pattern of the amino acids in urine in congestive heart failure.



Double dimensional chromatographic pattern of the amino acids in urine in congestive heart failure; sixteen ninhydrin-positive spots could be seen and fourteen of these could be recognized as particular amino acids.

Recent studies of Elias and Popper (1955) further explain the case with which central necrosis is produced in man contrasted with the rat. In rat, the periportal area in a particular lobule receives scanty blood supply from the main portal branch, in contrast to man in whom the areas around the middle-sized and larger portal triads receive blood directly from the main portal branch. There are similar differences in the drainage also. In rat, the drainage is free, sinusoids entering almost all branches of the hepatic vein. In man, only the central veins receive the sinusoids. The difficulty of lobular drainage is further aggravated by throttle mechanism produced by a fine arborization of the hepatic vein branches. Thus, in man as contrasted with the rat, the blood supply to the lobule from the portal tract is relatively free but the drainage is deficient. Hence, on the slightest provocation in the form of low cardiac output, anoxia or shock, central necrosis is produced. It then appears likely that any fall in the cardiac output, specially if accompanied by shock of a few hours duration, would lead to centrilobular necrosis. This, however, could not be the sole factor. Sherlock (1951) has reported that there was a group of patients with high cardiac output and who also had centrilobular necrosis. It is possible that in these cases, the high cardiac output may be associated with a low oxygen saturation. Anoxia itself may also cause a sinusoidal dilatation even in the absence of any mechanical obstruction to the blood flow (Seneviratne, 1949).

The finding of dilated central veins and dilated sinusoids in the central zone indicate increased pressure in the hepatic venous system. By and large this has been found to be true, as usually, the patients with constrictive pericarditis have the

maximal necrosis. However, in Sherlock's (*loc. cit.*) studies, no constant relationship was found to exist between the right auricular pressures and the degree of central necrosis. Another factor which contributes to the degree of necrosis is the duration of the failure as is obvious from our studies.

A full-fledged cardiac cirrhosis is relatively rare, probably due to the fact that prolonged recurrent episodes of circulatory failure are required for the laying down of connective tissue bands and distortion of the lobular architecture. Most patients die in the stages of early reticular condensation. It might appear that the cirrhotic lesions result from a replacement of the centrizonal necrotic tissue with connective tissue, the resulting capillarization establishing vascular shunts. This has been contradicted by the studies of Moschovitz (1952) who observed that congestive failure without prolonged hypertension got no further than extensive centrilobular necrosis and reticular condensation. In patients in whom portal hypertension of a central nature is maintained for a length of time varying degree of fibrosis occur, varying from sclerosis of central veins to the development of a full-fledged cirrhosis including central as well as portal fibrosis. His contention is based on the argument that histological evidence of 'Congestive splenomegaly' was present in every case. According to him cardiac fibrosis is not a simple replacement fibrosis but an active proliferative process. Vascular shunts are established between the portal and hepatic venous systems which serve to equalize the pressures.

Our own studies bear out that in prolonged congestive failure there is appreciable degree of histological and functional damage, though the liver still retains a fair degree of functional reserve and is capable of complete recovery with improvement in the circulatory failure. Nevertheless, repeated attacks particularly with mitral stenosis, bring about irreversible cirrhotic changes in the liver and once vascular shunts are established, the damage may be irreversible or even be progressive. We are not in a position to substantiate the theory of Moschovitz (*loc. cit.*) that a prolonged portal hypertension only can produce a cardiac cirrhosis as splenic enlargement was not noted to be a uniform observation in all cases of cirrhosis and the splenic tissues were not available for histological study.

The correlation of the clinical severity, the extent of central necrosis and the function tests also indicate that a clinical judgement may be a more accurate measure of the extent of damage to the liver than the function tests alone. There are no specific clinical or functional indications of cardiac cirrhosis except that a case with repeated episodes of long duration and particularly in the presence of mitral stenosis may be suspected to have developed fibrotic changes.

The observation regarding the amino-aciduria is interesting. The fact that the total number of amino acids for the same volume of urine increased from 3 to 16 in cases with moderate to marked congestive failure indicates that the amino-aciduria is an important metabolic change to be reckoned with. The exact mechanism of this change cannot be explained. As in other conditions, such as viral hepatitis where a breakdown of cells is going on, it may be speculated that here too, the amino-aciduria may be due to either a diminished capacity of the liver to deaminate the amino acids or due to an increased breakdown of the cellular proteins into amino acids. As the liver cells regenerate with diminished load of circulation on the liver the amino-aciduria also undergoes a notable diminution.

SUMMARY.

1. A combined clinical and biochemical study on 61 cases of different etiological types of congestive heart failure has been done. Out of these histopathological correlation was done in 29 cases in which puncture biopsy of the liver was done.

2. Histopathological changes showing the transition from the earliest centrilobular congestion to advanced cardiac fibrosis have been described.

3. Most severe form of central necrosis was seen in congestive heart failure due to mitral stenosis. The clinical severity of the failure was fairly closely related to the severity of central necrosis. The latter was also closely related to the duration of the disease. Mitral stenosis was also the most common cause of cardiac fibrosis.

4. Abnormality of the liver function tests was not closely related to the clinical severity of the disease. Albumin and globulin content, cephalin cholesterol flocculation and bromsulphalein excretion tests tended to be more abnormal in cases with advanced central necrosis. Patients with high serum bilirubin values were also those with severe hepatic damage.

5. A very significant finding was a marked amino-aciduria in patients with advanced congestive failure.

6. The mechanism of central necrosis and fibrosis is briefly discussed.

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* This reference has been consulted but not cited in the text.

EFFECT OF HYPOTHERMIA ON THE ACETYLCHOLINE AND GLUTATHIONE CONTENT OF BRAIN AND HEART OF DOGS.

BY

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HYPOTHERMIA offers new prospects in the treatment of congenital heart disease, cerebral vascular tumours, and aneurysms. There are, however, two significant hazards which handicap this advancement. The reduction of body temperature sometimes induces serious cardiac arrhythmias, particularly ventricular fibrillation, and irreversible changes in the nervous system, especially in the heart regulating mechanism, the respiratory, and cardiovascular centres. A fair amount of progress has been made to understand the mechanisms of these derangements and the treatment for it but the problem still remains to be completely solved.

These complications have variously been assigned to hypocarbia (Bigelow *et al.*, 1950), hypercarbia (Fleming, 1954; Lynn *et al.*, 1954; Swan *et al.*, 1953), hyperkalæmia (Sealy, 1954), hypokalæmia (Swan *et al.*, *loc. cit.*), too much calcium (Lynn *et al.*, *loc. cit.*), too little calcium (Swan *et al.*, *loc. cit.*), anoxia (Lutz, 1943), and acetylcholine metabolism derangement (Hegnover, 1949; Milch *et al.*, 1951; Noell, 1945).

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The views are extraordinarily conflicting to know all the details about the difficulties. The problem in this paper has been tackled by estimation of acetylcholine and glutathione in the hearts and brains of normal and hypothermic dogs.

METHODS.

Twenty normal dogs were used in this study. Six of these were used as controls, while in 14 hypothermia was produced.

The control animals were anaesthetized with ether and their heart and the following portions of brain removed:—

- (a) Whole of the hypothalamic region.
- (b) Anterior portion of the frontal lobe.
- (c) Portion of temporal lobe including the periamygdaloid region and the Ammon's Horn (hippocampus).

Acetylcholine was extracted and assayed from the heart and the brain portions by the method recommended by Nachmansohn and described elsewhere (Anand, 1952). Reduced glutathione (GSH) content of these samples was determined by the method of Hess as modified by Woodward and Fry (1952). From the values obtained in these control animals, normal averages of acetylcholine and glutathione content of heart, hypothalamus, frontal and temporal lobes were worked out.

In 14 dogs hypothermia was produced under ether anaesthesia. Shunt of the blood from femoral artery to femoral vein through a polyethylene tube, dipped in ice, supplemented with surface cooling, was used for producing this effect. Heart and brain portions were removed during the hypothermic condition of the animals, and the acetylcholine and glutathione content of the heart, hypothalamus, frontal and temporal lobes estimated.

The values for acetylcholine and glutathione obtained under hypothermic conditions were compared with the average normals obtained from control animals.

RESULTS.

The results of the acetylcholine and glutathione estimations in normal as well as hypothermic dogs are given in Tables I and II. It will be seen that acetylcholine content of the heart is lower than that of the nervous tissue, and that the hypothalamic region contains an appreciably higher level of it in comparison to the frontal and temporal lobes. Glutathione content also is lower in the heart as compared with the nervous tissue, but its concentration in the hypothalamus is about the same and not much higher as compared with the frontal and temporal lobes.

Acetylcholine was estimated in all the 14 dogs after hypothermia, while glutathione was determined only in 7 of these. An average fall of 14.5° C. below that of average body-temperature was obtained by the hypothermic techniques used. On comparing the average values of acetylcholine and glutathione after hypothermia with normals obtained from controls, it is clearly established that hypothermia decreases the amounts of both acetylcholine and glutathione in the heart,

and the hypothalamic and frontal regions of the brain. The decrease of these chemicals in all the three regions is statistically significant.

TABLE I.

Acetylcholine content of tissues of six normal and fourteen hypothermic dogs.

Normal dog: Average body temperature = 38.5° C. Hypothermic dog: Average body temp. = 23.9°C Results are given in μ per 1 g. of tissue.

HYPOTHALAMUS:		FRONTAL LOBE:		TEMPORAL LOBE:		HEART:	
Normal.	Hypothermic.	Normal.	Hypothermic.	Normal.	Hypothermic.	Normal.	Hypothermic.
0.368 $\pm$ 0.028*	0.297 $\pm$ 0.033	0.123 $\pm$ 0.007	0.113 $\pm$ 0.011	0.137 $\pm$ 0.006	0.268 $\pm$ 0.024	0.065 $\pm$ 0.005	0.053 $\pm$ 0.008

*Mean $\pm$ standard error.

TABLE II.

Glutathione content of tissues of six normal and seven hypothermic dogs.

Body temperature of dogs same as in Table I. Results in mg. per 1 g. of tissue.

HYPOTHALAMUS:		FRONTAL LOBE:		TEMPORAL LOBE:		HEART:	
Normal.	Hypothermic.	Normal.	Hypothermic.	Normal.	Hypothermic.	Normal.	Hypothermic.
1.107 $\pm$ 0.046	0.829 $\pm$ 0.031	1.050 $\pm$ 0.078	0.927 $\pm$ 0.061	0.877 $\pm$ 0.025	1.005 $\pm$ 0.055	0.387 $\pm$ 0.012	0.344 $\pm$ 0.024

It is, however, most interesting to note that hypothermia actually increases both the acetylcholine as well as glutathione content of the temporal lobes. The portions of the temporal lobe tested included the important limbic periamygdaloid and hippocampal (Ammon's Horn) regions. The possible significance of this observation is great and is discussed.

DISCUSSION.

Acetylcholine as a chemical transmitter of cholinergic impulses has been finally established since long (Minz, 1955). Its effect on the neuro-muscular junction has been approved to be utilized as a biological test for its detection (Minz, *loc. cit.*).

It has been determined that there is an optimum quantity of acetylcholine which is necessary for the physiological effects of this chemical in various regions of the central nervous system. This quantity is different in different tissues and varies with the embryological and functional development of the tissue. In the adult central nervous system it is maximum in the basal ganglia and minimum in the cerebellum, thalamus contains more than the hemispheres and grey matter more than the white matter. The most important quantities are present in the ganglia and fibres of the autonomic nervous system (Minz, *loc. cit.*).

In addition to its function as a cholinergic transmitter, acetylcholine also protects the tissues against anoxæmia. This it does in collaboration with glutathione which acts because of the role it plays in glycolysis during the anærobic metabolism and also because it accelerates the formation of more acetylcholine.

Glutathione is known to activate the cerebral phosphorylase in the concentration from 10 to 20 mm. (McIlwain, 1955). Damage to the sulfhydryl system by radiation or by chemicals in the embryo, which predominantly depends on anærobic glycolysis for its development and metabolism, is usually lethal (Hicks *et al.*, 1955). In hypothermia too the threat of anoxæmia is met by the cell by resorting to anærobic glycolysis with the help of its sulphhydryl system.

The way glutathione acts to affect the synthesis of acetylcholine has been worked out recently (Minz, *loc. cit.*). The acetylcholine cycle consists of splitting of this compound to choline and acetate by cholinesterase and then its re-building for its storage and liberation. The re-synthesis occurs in two steps. The first leads to the formation of 'active acetate' which is identical to co-enzyme A. The second step consists of active acetate combining with choline to form acetylcholine. The sulfhydryl group plays an important rôle in the first step, for co-enzyme A is acetylated on the sulfhydryl group of that enzyme. This work has established the intimate interdependence of glutathione and acetylcholine for their functional activity.

In the experiments it has been found that during hypothermia there is almost *pari passu* diminution of acetylcholine and sulfhydryl in frontal lobes, hypothalamus, and heart. During hypothermia there is diminution of the cell metabolism in the heart and nervous tissue which is reflected in the diminution of these two chemicals also. It is surmised that in cases where the diminution effects adversely, the mechanism to maintain the optimum quantity in the heart and the vital centres of the brain gets irreversibly damaged. This leads to the consideration and suggestion that it may be beneficial to give adequate doses of acetylcholine and glutathione during hypothermia as a prophylactic to prevent abnormal rhythm in heart and failure of the vital centres in the nervous system. This should be resorted to especially during recovery phase when the temperature begins to rise, the metabolism in the tissues is increased and there is likelihood of the development of anoxæmia.

In this experimental study the acetylcholine and glutathione contents of the temporal lobes containing Ammon's Horn (hippocampus) have been found to increase with hypothermia in contrast to what is found in the other regions of the brain. This signifies that the metabolic pattern of this region is not allied to the other tissues examined in this study. Ammon's Horn (hippocampus) is essentially a part of the limbic system whose function is primarily autonomic or visceral. The morphologic appearance and the biochemical constitution of this region has been investigated recently (Lowry *et al.*, 1954) and it has been pointed out that it differs from other regions of the cortical surface, such as the frontal and occipital lobes in several respects. Periamygdaloid region, including Ammon's Horn, is especially found to be related to the maintenance of body-temperature in general and temperature of the viscera in particular (Feindel *et al.*, 1954; Strich, 1956). It is tentatively surmised, therefore, that the increased metabolic activity of the periamygdaloid region may be because this region becomes hyperactive to counteract the hypothermia to maintain the normal body and visceral temperature. The

failure of this activity in this region may finally damage all ability on the part of the animal to regain normal body-temperature after the induction of hypothermia has been stopped.

SUMMARY.

1. Acetylcholine and glutathione estimations of heart, hypothalamic region, frontal lobe, and temporal lobe (including periamygdaloid and hippocampal regions) were carried out in 6 control dogs and 14 dogs made hypothermic.

2. With an average drop in the body-temperature of about 14.5°C., the acetylcholine and glutathione amounts significantly decreased in the heart, hypothalamus, and frontal lobe.

3. Hypothermia, on the other hand, increased the acetylcholine and glutathione content of the temporal lobe.

4. Significance of these observations is discussed and a suggestion made for the prophylactic use of acetylcholine and glutathione in hypothermia.

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EXPERIMENTAL PEPTIC ULCERATION.

Part I.

THE SIGNIFICANCE OF 'MUCOUS BARRIER'.

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THE importance of mucous secretion as a response of gastric mucosa to trauma has long been recognized (Bernard, 1856; Kaufmann, 1908; Whitlow, 1920; Babkin, 1928; Kim and Ivy, 1931). Florey and Webb (1931) investigated the protective rôle of mucous on gastric mucosa. Babkin *et al.* (1941) studied the secretory activity of the gastric mucous glands caused by the application of irritants. Wolf and Wolff (1943) observed in a patient of gastric fistula that the gastric mucosa which lay exposed had defective mucous formation, hence developed erosions. They further observed that if the mucous was wiped away, the mucosa became susceptible to irritants and bleeding rapidly occurred. Hollander, Stein and Lauber (1946) studied the secretion of mucous of Heidenhain pouches and noted that the more irritant the stimuli, the greater was the secretion of gastric mucous. Sanchez-Palomera (1951) suggested that the defence mechanism of gastric mucosa was superior to that present in other regions of gastro-intestinal tract.

Gastric mucous, which is secreted by the mucous cells, has been defined by Babkin (1950) as an independent tissue composed of carbohydrates, protein, mucoitin-sulphuric acid and hydrochloric acid. Ihre (1938), however, pointed out that 'all the fluid which arises from the stomach and which can act as dilutor, acid-binder or neutralizer of the primary acidity of the gastric juice' represented gastric mucous. Webster and Komarov (1932) cited two types of gastric mucin, one 'visible mucin' secreted by surface epithelium and the other 'dissolved mucin' obtained from the tubular gastric glands. The acid-binding power of mucous has been studied by Bolton and Goodhard (1933) and Boldyreff (1936). These workers have shown direct neutralization of acid by mucous. Inhibition of peptic

digestion by mucous has been demonstrated by Osterberg *et al.* (1933) and Bradley and Hodges (1934). With this in view, the rôle of mucous in the normal and patients of peptic ulceration was investigated, but the reports on the comparative concentration of gastric mucin were contradictory; therapeutic trials following administration of mucin to patients of peptic ulceration were also inconclusive (Babkin, 1929; Miller and Dunbar, 1933; Block and Rosenberg, 1933; Hurst, 1933; Anderson and Fogelson, 1936; Necheles and Coyne, 1938; Brumer, 1946; Tulin, Gutmann and Almy, 1947; Glass and Boyd, 1950). The significance of mucous barrier as a defence mechanism in relationship to peptic ulceration still remains to be investigated. In the present communication, therefore, an attempt has been made to study the relationship of mucous to experimental peptic ulceration.

MATERIALS AND METHODS.

Animals.—Male guinea-pigs of average weight 500 g. were used.

Production of mucous barrier.—*Capsicum minimum* Roxb.* an important flavouring and garnishing agent, has been used as an 'irritant' for mucous production. The dry pods of capsicum were ground in an electrically-driven grinding machine and sieved through a fine muslin. The sieved powder (0.5 g.) was then suspended in 10 c.c. of distilled water and used as a meal for the stimulation of mucous.

The gastric contents of guinea-pigs were aspirated at 10 a.m. Six and eighteen hours after aspiration 10 c.c. of capsicum suspension was introduced respectively through a No. 5 rubber catheter into the stomach. No food was given during the period of experiment and water was also discontinued from 6 a.m. on the day of injection until all the animals were sacrificed at 4 p.m.

Production of peptic ulcer.—The method of Halpern and Martin (1946), with suitable modification, has been found satisfactory. The guinea-pigs were injected 1 c.c. (5 mg./c.c.) solution of promethazine hydrochloride 'phenergan' (May and Baker) and after fifteen minutes 1 c.c. of histamine hydrochloride (E. Merck) solution (50 mg./c.c.) was given intraperitoneally. This was followed fifteen minutes later by another injection of the same dose of phenergan. The second injection of phenergan was a modification on the method of Halpern and Martin (*loc. cit.*). It was used to offer good protection to the animals against histamine toxicity. This method produced ulceration within a period of four hours.

PLAN OF THE EXPERIMENT.

The animals were divided into five groups and in each, gastric secretion and histopathological changes were investigated. Groups II, III and IV included 12 guinea-pigs each, while group I had only 6, and group V, 26 animals. In groups III and V, mucous barrier was produced as described, while in groups I,

*The dry pods contain about 0.1 per cent of a pungent principle, capsaicin ($C_{18}H_{27}O_3$) which is present in the inner wall of the fruit. The contents of capsaicin in this variety is within a narrow range, the average being 0.14 per cent (British Pharmaceutical Codex, 1934, Pharmaceutical Press, London and the Extra Pharmacopoeia-Martindale, 1941, The Pharmaceutical Press, London).

II and IV, instead of irritant suspension, 10 c.c. of distilled water (control) was administered. In groups IV and V the injections for the production of peptic ulcer were commenced one hour after the second meal. In group II in which the action of phenergan was alone tested, histamine was replaced by distilled water injection. All the animals in each group were sacrificed by ether anaesthesia four hours after the second meal.

Basal secretion of gastric juice was also studied in twelve normal guinea-pigs after fasting them for 24 hours.

HISTOPATHOLOGICAL TECHNIQUE.

Autopsy was performed on all the animals which were sacrificed including those which died during the injection period. After opening the abdominal cavity, the stomach was inspected *in situ* for motility, distension, congestion or perforation. It was then taken out, a slit of about an inch made from the duodenum along the greater curvature and the contents drained in a beaker. The stomach was then opened along the whole length of the greater curvature, pinned on a cork mat and fixed in 10 per cent formal saline for two hours to prevent autolysis. The degree of ulceration was then studied with a magnifying glass by vertically holding the stomach against light and lesions graded*, maximum score being 100: (a) hæmorrhage and erosion—20, (b) superficial ulceration showing breach in the mucosa—40, (c) deep ulceration where all the layers except a thin outer layer (serosa) intact—80, (d) perforation—90, and (e) extent of the surface involved—10. The ulceration indices were calculated according to the method of Pauls, Wick and McKay (*loc. cit.*).

The stomach was again pinned on a cork mat and fixed for four days after which one block each was selected from the cardiac, fundal and pyloric regions of the stomach. After complete fixation, the blocks were embedded in paraffin and cut at 5 μ . Three serial sections were mounted from each block, the first was stained with hæmatoxylin and eosin, the second with the PAS for mucin (Pearse, 1949), and the third kept as spare. The blocks which were fixed in Regaud's fluid after complete fixation, were also embedded in paraffin and two sections from each were cut at 5 μ . Pepsinogen was stained by Bensley's neutral gentian stain (Cowdry, 1948).

BIOCHEMICAL TECHNIQUE.

The gastric contents from each animal were centrifuged to remove any food particles present. After suitable dilution mucin was estimated as glucuronic acid (Dische, 1947). Free and total acidity was titrated with 0.01 N-NaOH using Topfer and phenolphthalein as indicators and expressed as m.eq/l and pepsin determined by the Mett-tube method (Hawk *et al.*, 1947).

*Pauls *et al.* (1947), Hay *et al.* (1942), Ambrus *et al.* (1953) and Brun (1952) found the grading of the severity and extent of the lesions effective and satisfactory and therefore, it has been employed in the present investigation.

RESULTS.

(a) *Biochemical.*

Normal animals.—The fasting contents of twelve normal guinea-pigs were studied for free and total acidity, peptic activity and mucin contents. The mean values and the range are summarized in Table I:—

TABLE I.
Gastric secretion of normal fasting guinea-pigs.

		VOLUME:	ACIDITY:		PEPTIC ACTIVITY:	MUCIN:
			Free.	Total.		
		c.c.	m.eq/l.		Mett unit.	Mg./100 c.c.
Mean*	...	2.2	53	92	8	114
Range	...	1.3-8.0	18-76	44-152	0.41	50-240

*Number of animals = 12.

Group I.—The effect of distilled water and rubber catheter on gastric secretion was studied and the results are shown in Table II.

Group II.—This group shows that phenergan has no action on mucous. Free and total acidity is of the same range as that found in water alone, but peptic activity appears to be depressed (Table II).

GROUP III.—In this group the main increase is of mucous the mean values of which are four times higher as compared to groups I and II. The free acidity and peptic activity are 2.8 and 1.4 times greater respectively, than seen in group I (Table II).

TABLE II.
Summary of results of biochemical investigations of gastric contents
(Groups I, II and III).

Group:		I	II	III
Number of animals:		6	12	12
Volume, c.c.	Mean	6.8	11.1	2.8
	Range	3.2-9.7	2.5-17.3	2.0-4.5
Free acidity, m. eq/l	Mean	10.5	7.3	28.2
	Range	0-21	0-28	7-115
Total acidity m. eq/l	Mean	22.8	24.6	74.8
	Range	12-38	11-47	24-130
Peptic activity Mett unit	Mean	3.5	0.5	5.0
	Range	0-22	0-4	0-36
Mucin mg. per cent	Mean	357	353	1431
	Range	288-448	224-560	887-2287

Group I water alone, group II water and phenergan, group III water and capsicum.

GROUP IV.—The mean values of acidity, peptic activity and mucin are shown in Table III. Negative correlation coefficient is found between mucin and free acidity. Two out of twelve animals, which died immediately after histamine injection, could not get the second injection of phenergan, hence were excluded from this group.

TABLE III.

Summary of results of biochemical investigations of gastric contents and degree of ulceration in guinea-pigs given water and injected with histamine and phenergan (group IV).

Animal number.	VOLUME.	ACIDITY :		PEPTIC ACTIVITY :	MUCIN :	DEGREE OF ULCERATION :		INDEX OF ULCERATION* :	
		Free.	Total.			Fundus.	Pylorus.	Fundus.	Pylorus.
	c.c.	m.eq/l		Mett unit.	Mg./100 c.c.				
1	14.0	5	16	0	245	0	0		
2	23.0	95	116	0	245	51	21		
3	18.5	60	75	0	216	71	0		
4	21.0	58	75	0	198	44	0		
5	23.0	55	78	0	245	42	82	44.4	25.3
6	24.0	35	53	27	245	71	0		
7	16.7	35	102	31	334	25	30		
8	13.7	18	52	0	384	65	65		
9	18.2	81	120	1	320	82	83		
10	12.7	75	102	0	256	42	0		
Mean	18.5	51.7	78.9	5.8	273	49.3	28.1		

*Index of ulceration = $\frac{\text{Mean degree of ulceration} \times \text{per cent of group ulcerated}}{100}$

Correlation coefficient. Mucin and free acidity = -0.25
 Correlation coefficient. Mucin and degree of ulceration = +0.08
 Correlation coefficient. Free acidity and degree of ulceration = +0.44

GROUP V.—The free acidity and peptic activity in group V (Table IV) is of the same order as noted in group IV. The mucous activity which is due to capsicum is three times higher as compared to group IV where no capsicum was administered. The correlation coefficient between mucin and free acidity is negative and statistically significant at 5 per cent level. The higher the mucin of the gastric juice, the lower were the values of free acidity.

(b) Histopathology.

Gross appearance.—The naked-eye examination of the stomach *in situ* of the normal, groups I and III, showed contracted stomach with prominent longitudinal folds on its surface. The mucosa was greyish pink and did not show any ulceration. In group II the stomach was congested and distended. Group IV showed ulceration of varying degree and extent in fundus and pylorus. In this group one animal had perforation and one showed normal appearance. The ulcerated animals had chocolate brown fluid in their stomach. Normal appearance of

stomach was noted in animals in group V where there was no ulceration, but the ulcerated ones had similar picture as seen in group IV.

TABLE IV.

Summary of results and biochemical investigations of gastric contents and degree of ulceration in guinea-pigs given capsicum meal and injected with histamine and phenegan (group V).

Animal number.	VOLUME. c.c.	ACIDITY:		PEPTIC ACTIVITY: Mett unit	MUCIN: Mg./100 c.c.	DEGREE OF ULCERATION:		INDEX OF ULCERATION*:	
		Free.	Total.			Fundus.	Pylorus.	Fundus.	Pylorus.
		m.eq/l							
1	7.5	32	96	6	1,225	0	0		
2	5.5	32	62	9	1,820	0	0		
3	20.0	67	114	11	700	30	0		
4	8.0	20	68	36	630	0	0		
5	5.0	23	90	15	1,120	0	0		
6	12.0	70	130	20	490	50	0		
7	3.0	85	155	33	630	20	0		
8	9.5	38	98	1	940	0	0		
9	10.8	92	115	25	274	21	0		
10	2.6	30	88	7	791	0	0		
11	37.5	82	120	0	566	82	41		
12	5.2	41	47	9	490	0	0		
13	8.5	45	68	4	274	23	0		
14	2.7	25	60	7	1,890	0	0		
15	12.5	20	38	0	1,575	0	0		
16	14.5	60	90	3	549	83	41		
17	2.7	22	58	0	802	0	0		
18	8.3	103	145	7	730	41	0	9.6	1.5
19	8.3	10	31	0	713	0	0		
20	28.0	99	168	0	774	21	0		
21	27.5	99	162	1	730	62	0		
22	10.5	12	35	0	1,028	0	0		
23	16.0	95	153	0	611	41	0		
24	10.5	74	121	0	967	0	0		
25	17.0	96	129	0	611	71	0		
26	8.5	31	43	0	610	0	0		
Mean	11.6	54	95.5	7.4	828	21	3.1		

* Index of ulceration = $\frac{\text{Mean degree of ulceration} \times \text{per cent of group ulcerated}}{100}$

Correlation coefficient. Mucin and free acidity = -0.45 (significant at 5 per cent level)
 Correlation coefficient. Mucin and degree of ulceration = -0.45 (significant at 5 per cent level)
 Correlation coefficient. Free acidity and degree of ulceration = $+70.0$ (significant at 1 per cent level)

The extent and degree of ulceration was noted and graded (Tables III and IV and, Plate III, figs. 1, 2, 3, and Plate IV, fig. 4).

Microscopic appearances.—The stomach layers of the normal animals did not show any abnormality. The acidophilic parietal cells showed bloated appearance,

the pepsinogen granules filled the peptic cells and the mucous cells were distended with mucin (Plate IV, fig. 5a). Groups I and II presented similar picture except marked congestion in the later. In group III, marked congestion was present in all the animals, and slight oedema, especially in the submucosa in two of them. The mucous cells showed hyperactivity, most of them were faint pink while others distinctly pink and few deep pink when stained with PAS. Some animals showed practically no mucin in the mucous cells (Plate IV, fig. 5b). At some places mucous was seen adherent to the superficial layer of mucosa. Pepsinogen granules appeared to be normal in amount. There was no depletion of the surface epithelial cells or any evidence of erosion, hæmorrhage or ulceration.

In group IV the erosion showed depletion of the superficial cells of the mucosa while the deeper cells remained intact. The submucosa had slight oedema, marked engorgement of blood vessels and diffuse infiltration in the ulcerated area with acute inflammatory cells mostly polymorphonuclear leucocytes (Plate V, fig. 6). The superficial ulceration showed complete destruction of the mucosa while muscularis mucosae, submucosa and serous layers were intact (Plate V, fig. 7). Deep ulceration was accompanied by the destruction of the muscularis mucosae and sometimes muscular layers. In one animal where perforation was noted, the margins of the perforation were everted and oedematous (Plate V, fig. 8). In this group marked congestion, oedema and infiltration with acute inflammatory cells was seen in the submucosa in all except one animal. Most of the animals showed dark pink mucous cells (PAS-stained) and normal amount of pepsinogen granules.

In group V the animals which did not have any ulceration showed marked activity of the mucous cells. In those animals where ulceration did occur the mucous cells were less active as evident from the staining reaction (PAS stain). The ulceration was of varying degree and presented similar picture as noted in group IV. The inflammatory cells seen in various layers of stomach in this group were comparatively fewer as compared to group IV.

(c) *Frequency of non-ulcerated and ulcerated animals.*

The number of non-ulcerated and ulcerated animals in groups IV and V is given in Table V. Their proportion is not independent of the groups, the animals in group V, however, showed statistically significant protection from ulceration.

TABLE V.

The number of ulcerated and non-ulcerated animals.

Group.	Non-ulcerated.	Ulcerated.	Total.
IV	1	9	10
V	14	12	26
Total	15	21	36

χ^2_1 (with Yates correction for continuity) = 4.05 $P < 0.05$

(d) *Comparative study of histopathological appearances and biochemical findings.*

The histological study of mucin in PAS-stained mucous glands and the mucin estimated in the gastric juice showed an inverse relationship. The degree of ulceration showed a negative correlation coefficient (group V) with the mucin content of gastric juice. The pepsinogen granules had similar relationship with the peptic activity but not in all animals. The higher the free acidity the greater was the degree of ulceration in the stomach and a positive correlation coefficient present which was statistically significant at 1 per cent level (group V).

DISCUSSION.

In the present experiments acute peptic ulceration has been produced within a period of four hours with massive doses of histamine in animals protected against its toxicity by phenergan. Mortality by our method was two in thirty eight animals which received massive doses of histamine as compared to one in every ten animals as reported by Brun (*loc. cit.*). The severity of the damage of the gastric mucosa varied from erosion to perforation. The mechanism of this ulceration has so far been not clear. Code (1943) suggested that the ulceration was the result of the corrosive action of acid in gastric juice secreted in response to histamine. Mann (1935) produced experimental gastric ulcers by continuous drip of 0.4 per cent of hydrochloric acid through a gastric fistula at the rate of one c.c. per minute for eight hours. Halpern and Martin (*loc. cit.*) presumed that the ulcers produced by the large doses of histamine in the animals protected against the toxic effects of histamine with antihistaminics were also due to the acid hypersecretion of gastric juice. Ivy, Grossman and Bachrach (1950) noted that if the maximum acid-stimulating dose of histamine was exceeded, the development of peptic ulceration was also accelerated and suggested that acid alone was not the answer. The experiments reported in this communication clearly demonstrate that the increase of free acidity was found to be directly correlated with the degree of ulceration. Besides the high acidity, congestion and oedema were other factors brought about by massive dosage of histamine. Merkel (1942) suggested that oedema caused by hyperemia and capillary stasis was responsible for the easy digestion of mucosa by acid gastric juice. It may, therefore, be suggested that in the present investigations hyperacidity and the oedema of the mucosa were the main factors in the causation of peptic ulceration.

Brun (*loc. cit.*) pointed out that the vascular effect of histamine cannot be separated from its secretory effects. Basu Mullik (1955) suggested that ischaemia led to ulceration. In his experiments ischaemia was produced by hypermotility which persisted for half an hour even after the animals were killed. Moeller and Kirsner (1954) also demonstrated that hypermotility was one of the causes for peptic ulceration. In our experiments the combined action of phenergan and high doses of histamine resulted in the delayed emptying of the stomach as evident by its bloated appearance. No visible peristalsis was seen at the time when the animals were sacrificed. The rôle of hypermotility in the causation of peptic ulceration, in the present experiment, however, cannot be substantiated.



FIG. 6. Early hæmorrhagic erosion involving only the superficial layer of the gastric mucosa. The blood vessels in the sub-mucosa are markedly congested. H & E $\times 30$.

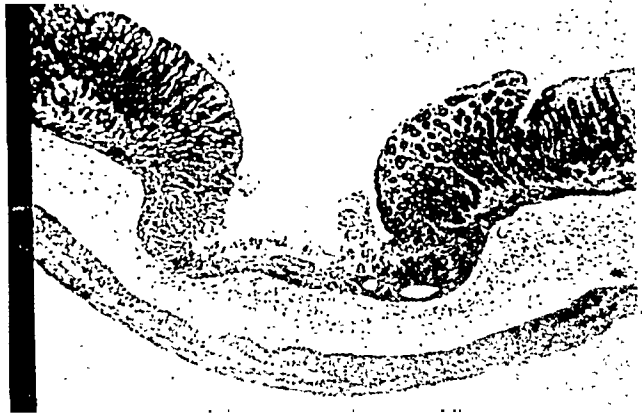


FIG. 7. The ulcer has involved the entire depth of the mucosa. H and E $\times 30$.



FIG. 8. Perforation. The margins are everted and œdematous. Right margin shows evidence of necrosis. H and E $\times 30$.

SUMMARY.

1. Acute peptic ulceration has been produced within a period of four hours with massive doses of histamine in guinea-pigs protected against its toxic effects by an antihistaminic-promethazine hydrochloride. The damage of the gastric mucosa varied from erosion to perforation accompanied by congestion and œdema. Significant positive correlation was found between free acidity and the degree of ulceration. The mechanism of ulcer production has been discussed. It has been suggested that hyperacidity and œdema of the mucosa were the causative factors for peptic ulceration.

2. The significance of mucous barrier in the prevention of histamine-induced ulceration has been studied. Mucous was produced in guinea-pigs by using capsicum as an irritant. The more the production of mucous, the less was the degree of ulceration. Mucous also protected mucosa and submucosa from inflammatory reaction. The higher the mucin contents, the lower was the free acidity. It has been suggested that mucin neutralized the action of acid gastric juice, also adhered to the surface of the mucosa and formed a barrier against peptic ulceration.

3. Capsicum in small doses for a short period did not produce any mucosal hæmorrhage or ulceration or even depletion of epithelial cells of the gastric mucosa of guinea-pigs. The most important gastric response was the production of massive amount of mucous.

4. Phenergan when administered to guinea-pigs brought about a delay in the emptying of the stomach. There was no change in the free acidity and mucin contents but the peptic activity appeared depressed. Phenergan did not induce hæmorrhage, erosion or ulceration of the gastric mucosa.

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INHIBITORY EFFECT OF STEROID HORMONES ON SALIVARY AMYLASE ACTIVITY.

BY

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IN the study of steroid enzyme relationships a working hypothesis held by some current investigators consists of a system in which hormones exert their action by direct or indirect effects on accelerators and/or inhibitors of enzyme systems. Hayano *et al.* (1950) have reported that desoxy-cortisone and other steroids strongly inhibit the d-amino acid oxidase system. Hochster and Quastel (1951) working on α -glycerophosphate dehydrogenase system found that the 3-ketosteroids possess the highest *in vitro* inhibitory activity, while 17- and 20-ketosteroids possess activity, although at a reduced level. Meyer and McShan (1950) reviewing various aspects of *in vitro* inhibition of the succinic-oxidase system have brought out that di-ethyl stilbestrol effectively inhibits this enzyme system in brain, adrenal, pituitary, kidney and heart tissue. The depressing *in vitro* action on the synthesis of acetylcholine in minced frog brain by steroids has been studied by Torda and Wolff (1950). This paper deals with the influence of testosterone and other steroid hormones on the salivary-amylase activity.

EXPERIMENTAL.

Specimens of saliva collected from human subjects, ages 18 to 25, were made use of in these experiments. The photometric method developed by Smith and Roe (1949) for the determination of amylase activity was employed to find out the amylase activity of each sample. Five c.c. of 1.2 per cent starch solution at approximately 90° C., 3 c.c. of phosphate buffer (pH 7.2) and 1 c.c. of 0.5 per cent sodium chloride were, respectively, transferred into each of two tubes labelled A and B, the latter serving as a control. They were placed in a water-bath at 37°C. until they attained the temperature of the bath. Afterwards 1 c.c. of diluted saliva was added to tube A, and both tubes were kept in the water-bath for 30 minutes. Two c.c. of normal hydrochloric acid was added to each tube promptly to stop amylase action in the digest tube and to prevent action of the enzyme next added to the control tube. One c.c. of the enzyme was added to the tube B. Next 2 c.c. of each of these mixtures were transferred to appropriately labelled 500-c.c.

40 *Inhibitory Effect of Steroid Hormones on Salivary Amylase Activity.*

flasks containing 400-c.c. distilled water and 5 c.c. NHCl . One c.c. of iodine reagent, prepared by dissolving 30 g. potassium iodide and 3 g. iodine in distilled water and making the volume to 1 litre, was next added to the two flasks, and the total volume made to 500 c.c. The resulting blue solution was decanted to cuvettes and read in Klett-Summerson photo-electric colorimeter at 620 $\text{m}\mu$. The colorimeter is initially set at 0 using 5 c.c. distilled water for blank and repeating the above procedure. The amylase units are calculated from the relationship:

$$\frac{(\text{Reading of the control}) - (\text{Reading of the digest})}{(\text{Reading of the control})} \times \frac{60}{10} \times \text{dilution} = \text{amylase units per c.c. of saliva.}$$

The results obtained for the normal values of salivary amylase activity for 11 subjects are tabulated in Table I:—

TABLE I.

Results showing normal range of amylase activity in human saliva.

Series.	Subjects.	Dilution.	Amylase units per c.c. of undiluted saliva.
1.	U.H.C.	1:100	460
2.	G.B.M.	1:100	552
3.	R.A.M.	1:150	675
4.	M.D.K.	1:150	965
5.	V.M.D.	1:100	625
6.	A.N.P.	1:150	515
7.	I.C.P.	1:100	492
8.	N.F.B.	1:100	480
9.	N.P.S.	1:100	462
10.	N.M.A.	1:150	558
11.	R.C.S.	1:150	738

Next, in order to study the influence of steroid hormones on amylase activity, the test steroid hormone* solution was added in known amounts, to the mixture of 5 c.c. starch substrate, 3 c.c. phosphate buffer, and 1 c.c. of 0.5 per cent sodium chloride and afterwards followed by the addition of 1 c.c. of dilute saliva and kept in a water-bath at 37° C. for 30 minutes. Repeating all the steps above described the altered amylase activity of the samples in the presence of the hormone was determined. The results obtained for four samples are recorded in Table II.

DISCUSSION.

The normal range of amylase activity in the saliva of the human subjects tested has been observed to be from 460 to 965 units; a unit of amylase being defined, in conformity with other units in vogue, as the amount of the enzyme that, under the conditions of this procedure, with 60 mg. of starch present, will

*The hormones were kindly donated by Unichem Laboratories, Bombay, through the courtesy of Mr. A. V. Mody.

hydrolyse 10 mg. of starch in 30 minutes to a stage at which no colour is given with iodine at 620 m μ . Inhibitory effects of both desoxycorticosterone acetate and progesterone on amylase activity can easily be noted by reference to Table II. The former brings about a nearly 5 per cent change in activity, while the latter a still higher change in activity to the extent of 9 to 11 per cent. It may also be said that this influence is in proportion to the increase in hormone levels, as the comparison of results in appropriate columns in Table II will show. Hayano and Dorfman (1951) have reported a slight inhibitory effect of desoxycortisone during the course of their work using commercial amylase. While the present results confirm their findings in respect of desoxycorticosterone, it will be observed that its inhibitory effect on human salivary amylase is greater and that in the case of progesterone its inhibitory influence is superior to that of desoxycorticosterone acetate. On the other hand, testosterone propionate shows activity but at a greatly reduced level.

TABLE II.

Results showing steroid hormone influence on salivary amylase activity.

Series.	Initial normal amylase units.	UNITS OF AMYLASE IN PRESENCE OF:								
		Desoxycorticosterone acetate mg./c.c. of starch.			Progesterone mg./c.c. of starch solution.			Testosterone propionate mg./c.c. of solution.		
		1.0	2.5	5.0	0.5	1.0	1.5	1.0	2.5	5.0
1	492	470	438	384	438	384	330	480	468	458
2	480	460	432	380	432	375	320	478	470	464
3	738	701	647	556	675	603	540	734	728	720
4	702	667	615	525	639	576	513	688	688	640

SUMMARY.

The normal amylase activity of human saliva and the inhibitory effects of desoxycorticosterone acetate, progesterone and testosterone propionate have been studied. The first two hormones have good inhibitory effect, while the last has a slight one.

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EFFECTS OF EGYPTIAN BLACK SNAKE TOXIN ON ASCORBIC ACID OF ADRENAL CORTEX AND ON EOSINOPHIL LEVELS.

BY

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WALTERINNESIA AEGYPTEA, or 'the Black Snake', which lives in the Sinai desert near Suez, is extremely poisonous. The snake was named after Walter Innes (1923) who was the first to discover the presence of the snake in Eastern Egyptian desert. Many fatal attacks by this snake upon camping soldiers and local inhabitants have been reported. Physiological effects of the purified toxin of the black snake have been recently studied by the authors. The changes observed in the blood picture (red cell count, total leucocyte and differential leucocyte counts), after injection of sublethal and lethal doses of the black snake toxin are similar to those which take place in conditions of hyper- and hypofunction of the suprarenal cortex, respectively (Mohammed and Zaki, 1956). In these circumstances it seemed possible that a more detailed investigation of the effect of the toxin on the adrenal cortex might prove to be profitable. This has been done by (a) examination of fluctuation in ascorbic-acid content of the gland, and (b) examination of changes in number of blood eosinophils.

The cortex of the adrenal has been shown by Szent-Györgyi (1928) to contain relatively large stores of ascorbic acid. The significance of the ascorbic-acid content has remained uncertain, but recent work has suggested that it is associated with or related to secretion of cortical hormones. It has been shown that the ascorbic-acid content of the adrenal is considerably reduced under the influence of low temperature (Sayers and Sayers, 1945), heat (Sayers and Sayers, 1947) and scalding (Ludwing and Chanutin, 1947). Sayers *et al.* (1946) showed that depletion of the adrenal content of ascorbic acid, when an animal is subjected to stress, could be produced by injections of anterior pituitary ACTH. In conjunction with the evidence of previous authors, this suggests that depletion of the adrenal content of ascorbic acid is an indication of increased output of cortical hormones. It is

evident from these facts that black snake toxin may induce changes in ascorbic-acid content of the suprarenal gland. The aim of this work is to examine the effects of the toxin on adrenal ascorbic-acid content, and to compare these changes with observed alterations in blood eosinophils.

METHODS.

A. *Changes in ascorbic-acid content of suprarenal gland.*—Extraction of the glands was done by the method described by Nasmyth (1950). Ascorbic-acid content of each extract was then estimated by the method described by Barakat, Fathy and El-Sadr (1955). For purposes of comparison, each value was calculated in terms of mg. ascorbic acid/100 g. gland tissue. The toxin used was prepared in purified form according to the method described by Mohammed and Zaki (*loc. cit.*) The toxin solutions in 0.9 per cent saline were freshly prepared for each day's experiments to avoid possible changes in properties on standing. The dissolved toxin was injected subcutaneously in the hind dorsal quadrant above the left thigh to come as close as possible to natural conditions of snake bite in man, which, in the majority of accidents, are inflicted in subcutaneous tissue layers. The toxin solutions were prepared at a ratio such that the total volume injected in each rat did not exceed one-half c.c. Albino rats of body-weight 150 g. to 200 g. were injected with sublethal dose (0.02 mg./100 g. BW) or lethal dose (0.05 mg./100 g. BW), killed at measured intervals and ascorbic-acid content of suprarenal gland was determined. In each experiment 5 rats received toxin by subcutaneous injection and 3 rats acted as controls. Each control was injected with 0.5 c.c. saline. The high dose levels of toxin were lethal after 1.5 hours; adrenal glands were removed before death for assay.

B. *Changes in circulating eosinophils.*—Blood eosinophil counts were carried out according to the Pilot (1950) method. Number of eosinophils was determined by counting total number present in the entire field of both sides of hæmacytometer. Three control counts were made on each animal prior to treatment.

RESULTS.

A. *Changes in ascorbic-acid content.*—In control experiments the normal content of the suprarenal cortex varied between 285 and 373 mg./100 g. gland (30 rats). The changes obtained after injection of toxin are given in the Table.

The sublethal dose caused diminution of the ascorbic-acid content of the adrenal gland. The maximal effect was attained 4 hours after injection of the toxin.

Examination of data on the action of lethal doses, revealed that the effects were not similar in all rats; in half of the rats (group I) there were no significant changes in ascorbic-acid content of the glands, whereas in others (group II), a slight preliminary reduction of ascorbic acid was followed by gradual rise above control levels.

B. *Changes in blood eosinophils.*—The results of typical experiments are shown in Table I. It is clear that the lethal dose of black snake toxin caused a marked increase in number of eosinophils. The sublethal dose caused an opposite effect:

gradual fall in number of circulating eosinophils until 6 to 10 hours after injection of the toxin, when the number began to recover to normal levels. In other experiments, lethal doses of toxin did not produce an eosinophilia in adrenalectomized rats.

TABLE.

Effect of sublethal and lethal doses of snake toxin on adrenal ascorbic acid content and on blood eosinophil levels of the rat.

	Number of animals.	Time interval.	Adrenal ascorbic acid.		Blood eosinophils.
Controls	30	Hour.	Mg./100 g. BW 325±13*		Aver. number/ field 8 (3 rats)
Sublethal dose of toxin (0.02 mg./100 g. BW)	5	1	205±42		5
	5	2	112±53		3
	5	4	106±27		2
	5	6	189±46		5
Lethal dose of toxin (0.05 mg./100 g. BW)	5	0.5	Group I.	Group II.	8 17 22
			310±26	212±37	
			299±32	382±51	
			342±37	455±55	

* Mean ± standard error.

DISCUSSION.

The sublethal dose of the toxin caused a significant depletion of adrenal ascorbic acid. The same dose of toxin also caused a gradual fall in number of circulating eosinophils. Abraham (1950) observed a correlation between degree of eosinopenia and depletion of adrenal ascorbic acid in conditions in which the animal was exposed to various agents which stimulated secretion of adrenocortical hormones. In accordance with the concept of the general adaptation syndrome, it has been postulated that an increased secretion of adrenocortical steroids occurred when an animal was placed under prolonged exposure to stress. Injection of the sublethal dose of toxin may be a type of such stress. The lethal dose caused variable effects. In some cases, no significant change in adrenal content of ascorbic acid was observed, yet in others there was an increase. The lethal dose, however, caused a marked eosinophilia. This was not, however, associated with a marked change in ascorbic-acid content of the adrenal glands of all rats. Increase in ascorbic-acid content of adrenals obtained in some experiments, therefore, cannot be considered as clear evidence of inhibition of the adrenal cortex. On the other hand, the marked eosinophilia obtained provides evidence suggestive of such an effect. It should be noted, moreover, that lethal doses of the toxin did not produce eosinophilia in adrenalectomized rats. Schwarz (1914), working on the nature and fate of eosinophil leucocytes, proposed the existence of endocrine stimuli for the regulation of their number in circulating blood. His suggestion was that

'diminished function of the adrenal glands should result in an increase, and hyper-activity in a decrease, of blood eosinophils'. This claim received corroboration from Abraham (*loc. cit.*), Abou-El-Naga and Saleh (1955), and Reinhardt and Shrewsbury (1955), among others, from the observation that adrenalectomy resulted in increase in number of circulating eosinophils.

SUMMARY.

1. A sublethal dose of Egyptian black snake toxin caused a marked eosinopenia, together with depletion of adrenal ascorbic acid in rats. Both effects are ascribed to hyper-activity of the suprarenal cortex, the toxin acting as a type of stress.

2. Injection of a lethal dose of toxin was associated with a significant eosinophilia, yet in most experiments there was no change or a slight increase in ascorbic-acid content of the adrenals. The eosinophilia obtained is interpreted as suggesting inhibition of the adrenal cortex.

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BLOOD GLUTATHIONE LEVELS IN THE DIABETIC.

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BLOOD glutathione levels are reported to be low in untreated *Diabetes mellitus* (Jizuka *et al.*, 1928; Kitamura, 1929; Varela *et al.*, 1930; Arnovljevitich, 1931; Labbe and Nepveaux, 1931; Bowman, 1934; Binkley *et al.*, 1950; and Illing *et al.*, 1951). Others, however, have reported normal values in this condition (Platt, 1931; Barbaro-Forleo and Cattaneo, 1935; Dogliotti and Meloni, 1935; Stock *et al.*, 1950; and Illing *et al.*, *loc. cit.*). Ralli *et al.* (1955) reported that although the difference in the -SH levels between the normal and the diabetic was not great, the median was lower in the diabetic subjects than in the normal. Lazarow (1949) put forth the hypothesis that lowered or insufficient concentration of a critical enzyme or co-enzyme (possibly GSH), or lowered or insufficient amounts of protective substance (again possibly GSH), or the inability or diminished ability to detoxify diabetogenic products (*of perhaps an altered purine metabolism*), increases the susceptibility of the beta-cells of the islets of Langerhans of the pancreas to the toxic action of these substances. Conn, Louis and Wheeler (1948) and Conn, Louis and Johnston (1949) noted a fall in blood GSH during adrenocorticotrophic hormone (ACTH) induced hyperglycemia. Shiv Kumar and Lal (1953) reported a lowering of blood GSH with a concomitant elevation of blood sugar in rats and rabbits under various experimental conditions. Hess, Kyle and Doolan (1951) noted this observation in man; but Sprague *et al.* (1950) did not observe any such change. According to Ogilvie (1952), susceptibility to the diabetogenic action of alloxan is inversely related to the sulphydryl content of the body. The present study was undertaken to determine whether or not the blood glutathione levels are altered in *Diabetes mellitus*. Twenty six cases of *Diabetes mellitus* were studied, and for purposes of comparison 94 normal, healthy adults were studied to serve as controls. Observations reported here suggest that the decreased concentration

GSH—reduced glutathione; GSSG—oxidized glutathione; Total—GSH+GSSG.

of GSH appears to be related to the presence of *Diabetes mellitus* and as compared to the controls, the values for GSH and total glutathione were appreciably lower in the diabetics.

MATERIAL AND METHODS.

Ninety-four apparently healthy adults from the staff and students of the Medical College, Amritsar, served as controls.

Twenty-six diabetics included in this study were cases admitted to the V. J. Hospital, Amritsar. The diagnosis in each case was confirmed by glucose tolerance studies. Blood glutathione and sugar values were determined in all normals and diabetics.

Procedure.—All blood samples were collected during 8 and 9 a.m., under basal conditions, during the post-absorptive phase, after half-an-hour's rest in the Laboratory in order to eliminate physiological or diurnal variations, if any. About 10 c.c. of blood were drawn from one of the arm veins, after a minimal stasis, with a clean, dry, all-glass syringe. Exactly 9 c.c. was immediately transferred to a test-tube containing 0.03 g. of finely powdered potassium oxalate. This provided sufficient filtrate for triplicate titrations for both reduced (GSH) and total glutathione.

Blood sugar estimations were performed in duplicate.

All estimations were performed immediately on collection. Blanks were run daily both for sugar and glutathione.

Methods.—Blood glutathione, both reduced and total, was determined by the iodometric titration method of Woodward and Fry (1932), with minor modifications as suggested by Blyakner (1938). The average thioneine error of this method would be 3 mg. per 100 c.c. blood.

Oxidized glutathione was determined by the difference in the analyses for reduced glutathione in samples before and after reduction of oxidized glutathione with zinc dust (Woodward and Fry, *loc. cit.*).

Blood sugar estimations were done according to the method of Hagedorn and Jensen (1923).

RESULTS.

In the normal controls (94 subjects), the blood GSH values range between 24.5 and 46.1 mg. per 100 c.c. blood; GSSG values range between 1.8 and 18.4; total glutathione between 28.8 and 53.7. The ratio GSH: GSSG ranges between 1.8 and 17.7. Blood sugar range is 70 to 100 mg. per 100 c.c. blood. The statistical constants are presented in Table I.

The relationship between blood sugar and blood GSH, GSSG and total glutathione (GSH + GSSG) was subjected to statistical treatment. The correlation coefficient for GSH is -0.2155 which is significant at 5 per cent level of significance. The test brings out the conclusion that GSH bears a negative significant correlation with blood sugar in normal persons, indicating that in normal persons a high content of blood sugar generally reflects a lower blood GSH level.

TABLE I.

Some statistical constants connected with blood sugar and glutathione in normal persons.

(Number of observations is 94.)

(All values are in mg. per 100 c.c. whole blood.)

Serial. number.	Statistical constant.	Blood sugar.	GSH.	GSSG.	Total.	GSH/GSSG.
1.	Range ...	70-100	24.5-46.1	1.8-18.4	28.8-53.7	1.8-17.7
2.	Mean ...	86.95	33.06	7.0	40.05	6.0
3.	Standard deviation ...	7.77	4.69	3.49	5.84	3.33
4.	Standard error of mean	0.8010	0.4835	0.3597	0.6020	0.3433
5.	Coefficient of variation	8.94	14.19	49.85	14.58	55.5

No significant correlation, however, exists between blood sugar and blood GSSG or with total glutathione of blood (GSH + GSSG).

The data and statistical constants of diabetes are presented in Table II. The relationship between blood sugar and the different blood glutathione fractions was also subjected to statistical treatment and no significant correlation could be demonstrated in the case of the diabetics between blood sugar and blood GSH, or GSSG or total glutathione (GSH + GSSG).

DISCUSSION.

Our figures are in agreement with those of Woodward and Fry (*loc. cit.*); Ceresa and Gualla (1939), Caren and Carne (1951), Illing *et al.* (*loc. cit.*), and Shiv Kumar *et al.* (1954). All of them utilized the iodometric method as applied to sulfosalicylic acid filtrates and the relative superiority of this method to the others available has already been referred to (Shiv Kumar *et al.*, *loc. cit.*). But, whereas Varela *et al.* (*loc. cit.*) and Ceresa and Gualla (*loc. cit.*) are of the view that individual variation in blood glutathione in normal, healthy conditions is relatively moderate, no such opinion can be expressed on the basis of the current study. The range, standard deviation and standard error of the mean show that even in a homogenous group, the variation is considerable; consequently, any deviation from the normal must be large if it is to be of any significance in *Diabetes mellitus*.

The lower blood glutathione values observed in human diabetics by the authors, are in agreement with the observations of Kitamura (*loc. cit.*), Varela *et al.* (*loc. cit.*), Campanacci (1930), Campanacci and Negri (1931); Arnoljevitch (*loc. cit.*); Labbe and Nepveux (*loc. cit.*), Bowman (*loc. cit.*), Pickard and Marsden (1934), Binkley *et al.* (*loc. cit.*). Others have reported normal values in diabetics, the more recent reports being those of Caren and Carne (*loc. cit.*); Illing *et al.* (*loc. cit.*), and Stock *et al.* (*loc. cit.*). There is one report, that of Paolini and Cucuzza (1928), which records raised blood glutathione levels in *Diabetes mellitus*,

COMPARATIVE ABSORPTION STUDIES OF BUFFALO BUTTER FAT (GHEE) IN ALLOXAN DIABETIC AND ETHIONINE-ADMINISTERED RATS.

BY

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INTRODUCTION.

PREVIOUS workers (Barbour, 1934; Basu and Nath, 1946; 1946a, Deuel and Holmes, 1921; Heilditch, 1941; Irwin *et al.*, 1936; Longworthy and Holmes, 1915; and Longworthy, 1923) have reported the absorption of fat and fatty acids and also the rôle of sex on the rate of such absorption. In the present investigation the rate of absorption of 100 g. of buffalo butter fat (ghee) which contained 14.88 g. weight of fatty acids in terms of stearic acid was undertaken in normal animals and compared with those having fatty livers induced by ethionine (Farber, Simpson, and Tarver, 1950; Farber, Kochweser and Popper, 1951; Jensen, Chaikoff, and Tarver, 1951) and with the alloxan diabetic animals.

EXPERIMENTAL.

For the purpose of this study about 30 adult albino rats of either sex, weighing approximately between 150 g. and 200 g., were used. These animals were divided into three groups. Group I—normal animals, group II—ethionine-administered female animals, and group III—diabetic animals. They were given fat-free standard diet* for 48 hours.

* The diet had the following composition:—

Casein	...	...	20 g.	Vitamin powder	...	1 g.
Dextrin	...	...	5 g.	Sucrose	...	69 g.
Salt-mixture	...	...	5 g.			

The animals were then fasted for 48 hours after which buffalo butter fat (ghee) was administered. During the period of fasting, only water was allowed to these animals. Butter fat (ghee) was administered by stomach tube and small amount of remaining ghee in the tube was recovered in each case and duly allowed for in calculating exact quantity introduced into the stomach.

The amount of ghee absorbed after 2, 4 and 6 hours was determined. At the end of these definite intervals, the rats were sacrificed. The intact gastro-intestinal tract was removed and fat remaining unabsorbed was determined by water and petroleum-ether extraction method according to Saxon (1952).

PREPARATION OF ANIMALS.

(a) *Ethionine-administered rats.*—Animals were fasted for a period of 24 hours after which 50 mg. of ethionine was injected intraperitoneally in 5 such doses at intervals of two hours. Following this, the animals were taken up for fat absorption studies.

(b) *Alloxan diabetic rats.*—Animals of either sex weighing 150 g. to 200 g. were used. The animals were fasted for 48 hours at the end of which period they were injected intravenously with alloxan monohydrate dissolved in saline in a dose of 10 mg. each. Following the injections, animals were restrained from feeding for a period of 30 minutes after which they were given regular insulin in the amount of four units per day. The animals were given free access to one per cent saline solution. Urine examination was undertaken in all these animals every day and only those exhibiting diabetes were undertaken for this study. Invariably the animals passed about 3 to 4 per cent sugar.

RESULTS.

Results obtained in different groups are represented in Tables I to III:—

TABLE I.

Normal rats.

Time in hours.	Amount administered 2 c.c.	Amount recovered from gastroint. tract.	Amount absorbed.	Percentage absorbed.	Mean.
2	1.8340	0.9060	0.9280	50.59	54.53
		0.7878	1.0462	57.02	
		0.7990	1.0350	56.50	
4	1.8340	0.6790	1.1550	63.00	63.08
		0.6532	1.1808	64.32	
		0.7110	1.1230	61.92	
6	1.8340	0.6650	1.1690	64.10	65.83
		0.6300	1.2040	66.20	
		0.6010	1.2330	67.20	

TABLE II.

Ethionine-administered rats.

Time in hours.	Amount administered 2 c.c.	Amount recovered from gastroint. tract.	Amount absorbed.	Percentage absorbed.	Mean.
2	1.8340	1.4392	0.3948	21.53	27.70
		1.2440	0.5900	31.17	
		1.2950	0.5390	29.40	
4	1.8340	1.2670	0.5670	30.91	38.63
		0.9760	0.8580	46.08	
		1.1210	0.7130	38.90	
6	1.8340	0.6532	1.1808	64.32	55.65
		0.8812	0.9528	51.90	
		0.9016	0.9324	50.73	

TABLE III.

Diabetic rats.

Time in hours.	Amount administered 2 c.c.	Amount recovered from gastroint. tract.	Amount absorbed.	Percentage absorbed.	Mean.
2	1.8340	1.5158	0.3182	17.26	17.48
		1.5636	0.2704	14.73	
		1.3590	0.4750	19.57	
4	1.8340	0.8376	0.9964	54.35	51.39
		0.9550	0.8770	47.92	
		0.8812	0.9528	51.90	
6	1.8340	0.7010	1.1330	61.77	58.84
		0.7440	1.0900	55.00	
		0.7390	1.0950	59.75	

DISCUSSION.

Higher percentage of fat absorption was observed with buffalo butter fat (ghee) in the present studies specially for 2 and 4 hours absorption when compared to the values of Basu and Nath (*loc. cit.*) who used cow butter fat. Similar results were observed even when the values were reduced on the basis of 100 g. body-weight.

Percentage absorption of buffalo butter fat was reduced in ethionine-administered rats having markedly fatty livers and in diabetic animals. This was quite marked for two and four hours periods. This points out how the optimum rate of absorption in the normals can be affected under different metabolic conditions.

Comparative Absorption Studies of Buffalo Butter Fat.

SUMMARY.

Fat absorption studies were undertaken in normals, ethionine-administered and alloxan diabetic rats for periods of 2, 4 and 6 hours, respectively. Fat absorption in normals was observed on an average to be 54.53, 63.08 and 65.83 per cent for 2, 4 and 6 hours' periods, respectively. Average absorption values of 27.67, 38.63 and 55.65 per cent were observed in ethionine-administered rats for the above-mentioned period, while the values for alloxan diabetic rats observed were 17.58, 51.39 and 58.84 per cent, respectively.

In ethionine-administered rats the absorption was reduced for two and four hours' period. In uncontrolled diabetic rats, there was a decrease in the absorption in two-hour period but no marked difference was seen for four and six hour periods.

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EFFECT OF 'NADISAN' ON THE COURSE OF ALLOXAN DIABETES IN RATS AND RABBITS.

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INTRODUCTION.

PUECH AND LOUBATIERES (1947) observed that sulphurous water caused improvement in alloxan diabetic rabbits. Houssay (1950) in a review article reported that administration of sulphur compounds which increase tissue glutathione either prevent or delay the onset of different types of experimental diabetes. Blood sugar lowering effect of several compounds of the nature of sulphonamide has been observed by Ruiz *et al.* (1930), Janbon *et al.* (1942), Loubatieres (1944, 1944a, 1946) La Barre and Reuse (1947), and Chen *et al.* (1946). Recently, a sulphonamide compound (Nadisan, BZ. 55) discovered in Germany has been claimed to be effective in bringing down blood sugar when administered orally. Findings on the trial of this new drug in clinical and experimental diabetes have been published by many workers (Franke and Fuchs, 1955; Bertram *et al.*, 1955; Achelis and Hardebeck, 1955; Mirsky *et al.*, 1955; Miller and Dulin, 1956; Bastenie, 1956; and Young, 1956). Therefore, to study the effect of this new sulphonamide compound 'Nadisan', on the incidence and course of alloxan diabetes in rats and rabbits, the following experiment has been carried out.

MATERIALS AND METHODS.

Healthy adult rats and rabbits of C.D.R.I. colony were used for our experiment. The animals were kept under observation on stock diet for a period of one week and their fasting (24 hours) blood sugar examined before starting experiment. One batch of 36 rats weighing approximately 130 ± 1.2 g. was divided into six groups of six rats each. Groups I, II and III rats were fasted for 18 hours. Nadisan suspended in water was forced-fed in a single dose of 0.25 g./kg. to each of

group I, 0.5 g./kg. to each of group II and 1 g./kg. to each of group III. Two hours after Nadisan feeding all the animals of the above three groups (still without food) received subcutaneous injection of alloxan monohydrate in 1 per cent solution in the dose of 175 mg./kg. each. Nadisan was fed to groups IV and V animals in a dose of 0.5 mg./kg. twice daily for three and six days, respectively. On the third and sixth day these rats (groups IV and V) were kept without food for 18 hours (but Nadisan was fed) and two hours after the last Nadisan feeding they were injected with alloxan monohydrate in the same dose and concentration as above. Group VI animals received subcutaneous injection of alloxan monohydrate only in the same dose and concentration and under similar fasting condition as above. These animals (group VI) served as alloxan diabetic control. Blood sugar (12 hours fasting) of all the above six groups of animals was estimated and their urine examined for sugar 48 hours after alloxan administration.

Another batch of 72 rats of average weight of 150 ± 7 g. received subcutaneous injection of alloxan in 18 hours fasting condition in the dose of 175 mg./kg., in 1 per cent solution. Several (38) of these animals died within seven days of alloxan administration in spite of forced feeding and soluble insulin treatment. Fasting (24 hours) blood sugar of the surviving 42 rats was determined on the seventh day after alloxan administration and the animals with hyperglycemia above 300 mg. per cent were divided into three groups—groups VII, VIII and IX consisting of 15, 15 and 12 animals, respectively. Nadisan feeding in dose of 0.5 mg./kg. daily was started to group VII rats after seven days of alloxan diabetes (acute stage of diabetes) and to group VIII rats after two months of alloxan diabetes (chronic stage of diabetes), 5 of the group VIII animals died during 2 months of alloxan diabetes. To both the groups Nadisan feeding was continued for 8 weeks. Group IX animals received no Nadisan and served as untreated diabetic control. Occasional insulin treatment (4 units per rat per day) was continued to group VIII animals to keep them survive for 2 months prior to Nadisan feeding. Animals were kept without food and insulin for 12 hours previous to every blood sugar estimation. Blood sugar was determined and weight was taken once every week of all the animals during experimental period.

Six rabbits weighing between 1.8 kg. and 2.2 kg. were fasted for 12 hours and were given intravenous injection of alloxan monohydrate in the dose of 175 mg./kg. in 1 per cent solution. To pass off the critical period the animals were treated with 20 units of clear insulin daily in two dosage for seven days. Animals were kept without food and insulin for 12 hours before their blood-sugar determination on the eighth day. Hyperglycemia in all the six animals was over 400 mg. per cent. Nadisan feeding in the dose of 0.5 g./day/rabbit was started to three of the animals from eighth day after alloxan diabetes (acute stage of diabetes) and to the other three from fourth month after induction of alloxan diabetes (chronic stage of diabetes). Nadisan feeding was continued daily for four weeks to all the six animals. Clear insulin 8 units/rabbit/day was administered occasionally to the latter three rabbits to keep them survive during 4 months prior to Nadisan feeding. Their blood sugar (without food and insulin for 12 hours) was determined and body-weight noted once every week during observation period. Blood-sugar estimation was carried out according to Folin and Wu's micro-method.



FIG. 2. Extensive hæmorrhage
and superficial ulceration $\times 1$.



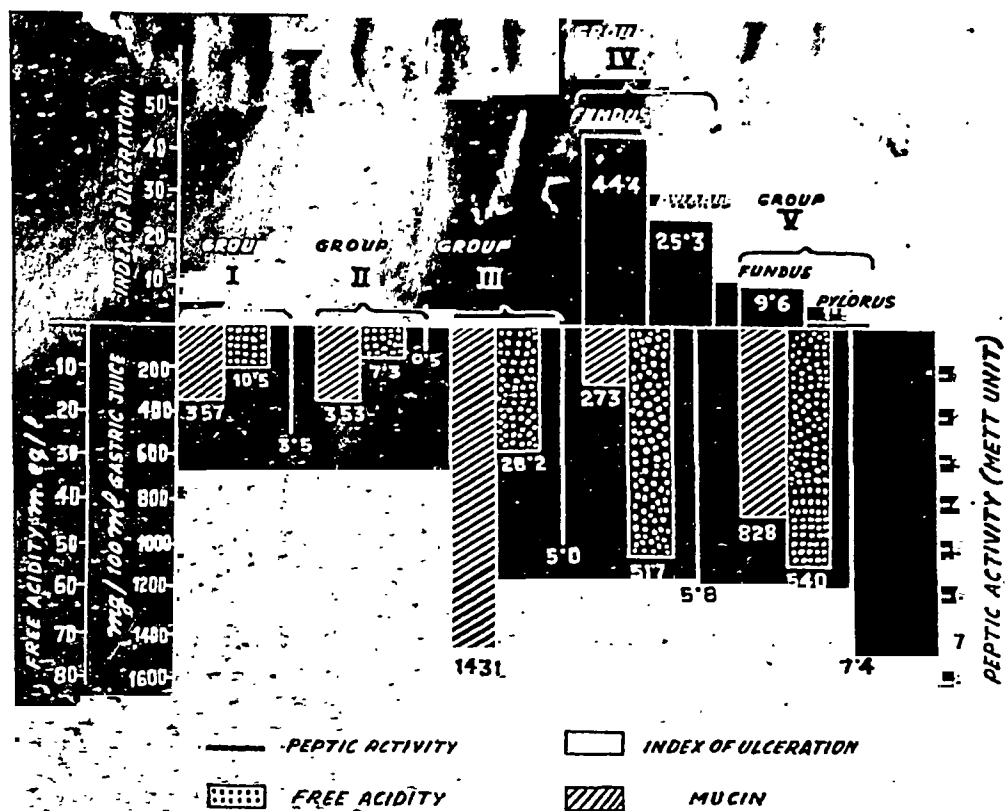


FIG. 4. Index of ulceration and mean values of gastric contents Gr. I. water alone, Gr. II. water and phenegan, Gr. III. water and capsicum, Gr. IV. water, phenegan and histamine and Gr. V. water, capsicum, phenegan and histamine.

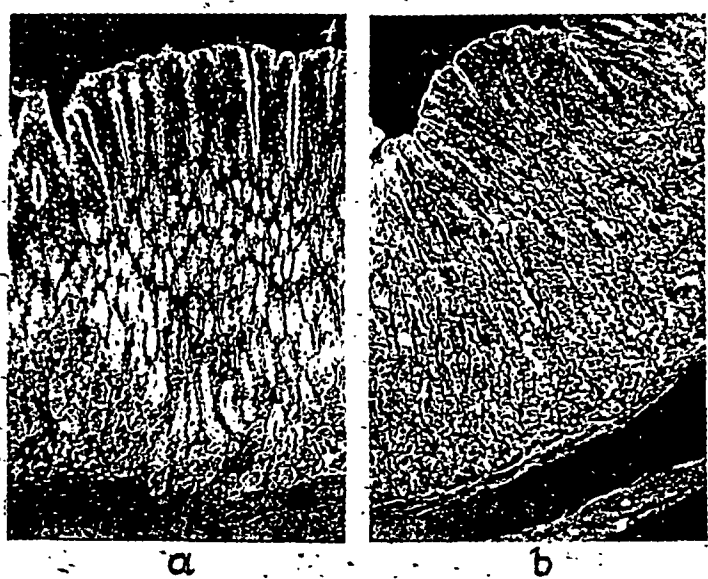


FIG. 5a, b. Normal gastric mucosa shows strongly PAS positive mucin in the mucous cells (Fig. 5a) which contrasts with practically negative mucin after capsicum stimulation (Fig. 5b) $\times 70$.

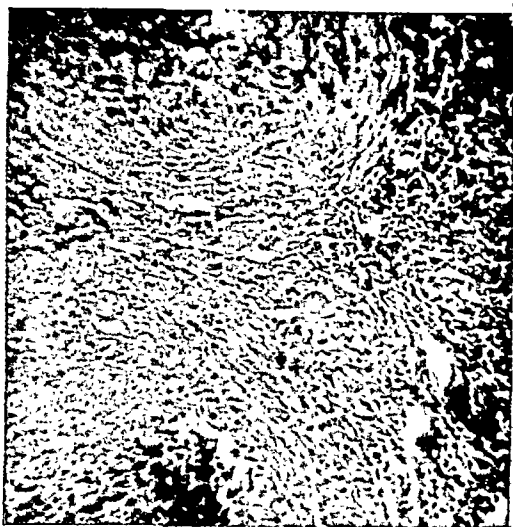


FIG. 5. Is from a section of the lymph nodes of a rat from the low protein (5 per cent) group showing collection of epithelioid tubercles with little or no evidence of necrosis. H & E $\times$

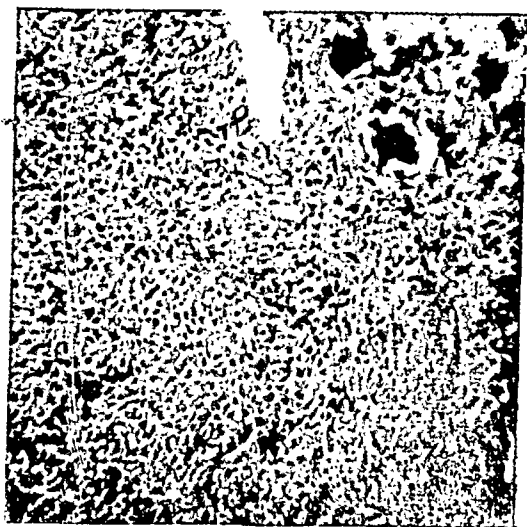


FIG. 6. Is from a section of the lymph node of a rat from the high protein (18 per cent) group showing a large epithelioid tubercle with tiny foci of necrosis. H & E $\times$

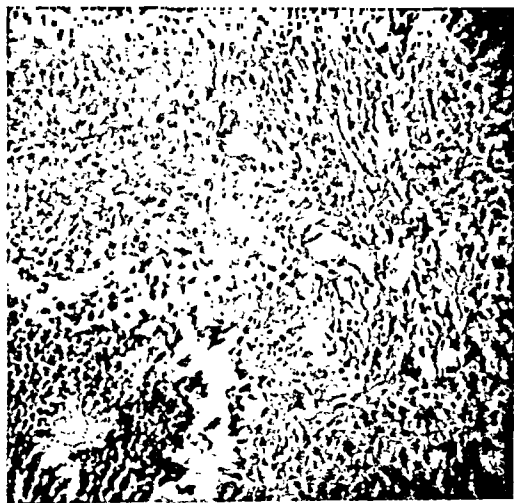


FIG. 7. Is from a section of the lymph node of a rat from the high protein calorie restricted group showing a large epithelioid tubercle—with central necrosis. H & E $\times$



FIG. 8. Is from a section of the lymph node of a rat from the high protein vitamin A deficient group showing an epithelioid tubercle with the dark area in the centre representing a large tuft of tubercle bacilli. H & E $\times$

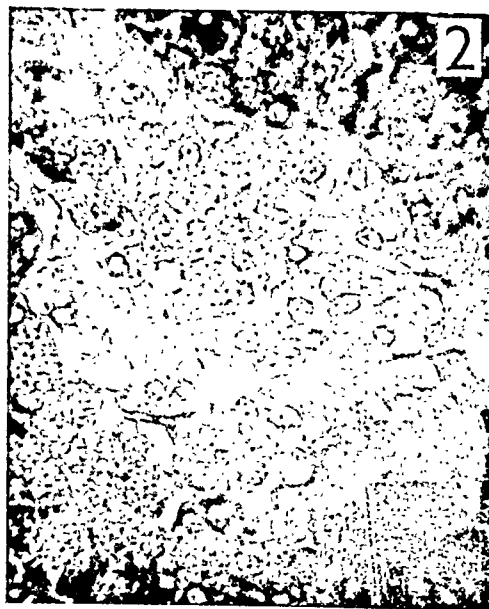
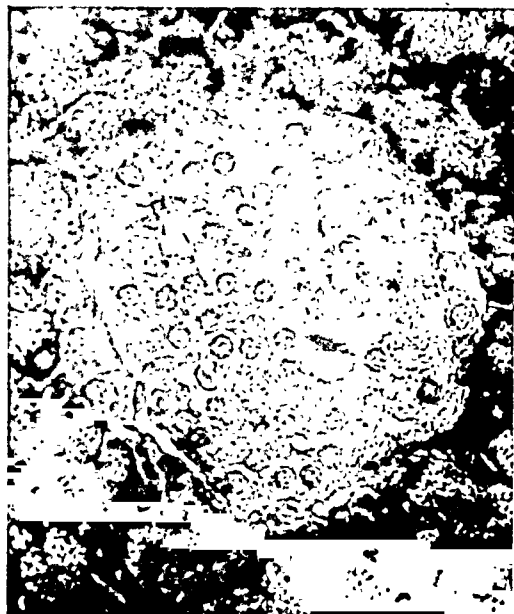


FIG. 1. Island of Langerhans of a normal rat $\times 470$.

FIG. 2. Island of Langerhans of an untreated diabetic control rat $\times 470$.

No β cells outline is seen. The cytoplasm of the remnants of β cells is highly degranulated. Shadows of few shrunken and irregularly placed nuclei can be seen.

FIG. 3. Island of Langerhans of a Nadisan-treated rat (acute stage of diabetes) $\times 470$.

β cells are hardly seen anywhere in the island. A dirty staining remnant suggesting complete necrosis of the β cells is present.

FIG. 4. Islands of Langerhans of Nadisan-treated rat (chronic diabetes) $\times 470$.

Only a few necrosed β cells are found in the centre of the island, However, in the peripheral zone a strip of coarsely granular cells (granules of cells are stained violet) with centrally placed nuclei are seen.

Pancreas of the rats at death during experiment and 8 weeks after Nadisan feeding was collected and the islands of Langerhans were studied by Gomoris (1939) chrome-alum-hematoxylin-phloxin technique as well as by routine hematoxylineoslin procedure.

TABLE I.

Effect of Nadisan feeding in single dose and in repeated doses prior to alloxan administration on the incidence of alloxan diabetes in rats.

Group and number of animals.	Dose and duration of Nadisan feeding.	Dose of alloxan injected, S. C.	Blood sugar mean average in mg. per cent 48 hours after alloxan injection.	Urine sugar 48 hours after alloxan injection.	Number of animals diabetic.
Group I. 6 animals	0.25 g./kg. single dose	175 mg./kg.	426±22	++++	6/6
Group II. 6 animals	0.5 g./kg. single dose	„	650±50	++++	6/6
Group III. 6 animals	1.0 g./kg. single dose	„	527±41	++++	6/6
Group IV. 6 animals	0.5 g./kg. twice daily for 3 days	„	498±10	++++	6/6
Group V. 6 animals	0.5 g./kg. twice daily for 6 days	„	525±45	++++	6/6
Group VI. 6 animals	No Nadisan feeding	„	575±24	++++	6/6

++++ Urine sugar above 2 per cent.

The above results (Table I) of blood sugar and urine sugar examined 48 hours after alloxan administration show that all the six groups of rats are in a severe state of diabetes. Hyperglycemia and glycosuria in both the Nadisan treated and untreated rats are of the same magnitude.

Results given in Table II show that the blood sugar in the two groups of rats which were fed Nadisan daily for eight weeks remain almost to the same high level throughout the period as in the untreated diabetic rats. Practically, no difference in the diabetic condition, hyperglycemia, glycosuria and progressive loss of body-weight, was noticeable in any of the three groups of rats. The result further show that Nadisan feeding started either after seven days or after two months of alloxan diabetes does not modify in any way the diabetic condition in the animal.

Results in Table III show that there is no tendency of the blood sugar in six rabbits to come down from the pre-treatment (diabetic) level either during or after Nadisan feeding. Presence of marked glycosuria and progressive loss of body-weight is also noticeable in all these animals.

TABLE II.

Number of animals.	BLOOD SUGAR IN MG. PER 100 G.G.:		Total loss of weight in g. at the end of observation period.	
	On 7th day after alloxan.	Mean average during and at the end of 8 weeks of Nadisan feeding.		
1	560	500±20	20	Died during experimental period.
2	420	390±30	19	
3	400	535±15	52	
4	310	412±41	52	" "
5	320	400±43	32	
6	360	325±25	74	
7	470	530±53	39	" "
8	420	275±50	40	
9	435	385±22	25	
10	360	395±25	28	" "
11	370	403±164	19	
12	400	402±57	30	
13	375	315±50	51	" "
14	385	495±45	13	
15	330	420±55	22	
Group VIII	On 58 days after alloxan.			
1	480	422±25	20	Died during experimental period.
2	380	332±41	22	
3	500	585±24	52	
4	380	410±94	32	" "
5	580	354±25	26	
6	500	400±35	25	
7	380	490±57	40	" "
8	470	551±42	24	
9	400	332±41	32	
10	390	300±42	29	
Group IX.				
12 animals	Mean average of blood sugar and loss of body-weight in 12 untreated diabetic rats during observation period.			
		476±44	27±7	5 animals died during experimental period.

DISCUSSION.

It is evident from results in Table I that Nadisan in a single dose or in repeated doses fed prior to alloxan administration does not prevent the diabetogenic action of alloxan in rats. Intensity of hyperglycemia and glycosuria in the Nadisan-fed groups of rats are of the same magnitude as in the untreated alloxanized rats. Our observations further show that continuous Nadisan feeding in acute and chronic alloxan diabetic rats and rabbits does not in any way ameliorate their diabetic condition. Degree of hyperglycemia and glycosuria and the

progressive loss of body-weight in the Nadisan-fed (diabetic) animals remain more or less the same as prior to Nadisan feeding. From histological study of the pancreas (Plate VI, figs. 2, 3 and 4) it is evident that the degenerative character of the islands of Langerhans in Nadisan-fed diabetic animals does not materially differ from those of untreated groups of diabetic rats. No evidence of stimulation of the existing cells is observed anywhere in the sections (Plate VI, figs. 3, 4).

TABLE III.

Effect of Nadisan feeding (0.5 g./kg. daily) on the course of acute and chronic alloxan diabetes in rabbits.

Number of animals.	BLOOD SUGAR IN MG. PER 100 C.G.:		Total loss of weight at the end of observation period, g.	
	On 7th day after alloxan.	Mean average during and at the end of 4 weeks of Nadisan feeding.		
<i>Acute</i>				
1	336	420 $\pm$ 40	250	Died during experimental period.
2	410	470 $\pm$ 57	150	
3	440	300 $\pm$ 50	200	
<i>Chronic</i>	On 120th day after alloxan.			
4	400	390 $\pm$ 36	180	
5	390	400 $\pm$ 49	200	
6	420	370 $\pm$ 50	290	

Acheles and Hardebeck (1955) observed that though some of their experimental animals responded to Nadisan feeding yet a number of animals did not respond to Nadisan feeding. Our observation tends to support only the negative findings of Acheles and Hardebeck. Our results appear to be in agreement with the recent observation of Kirtley *et al.* (1956) who studied the effect of sulphonamide (BZ55) on various species of alloxan diabetic animals (rats, rabbits and dogs) and of Mirsky *et al.* (1956) and Bender and Scholz (1956) who also studied the effect of another sulphonamide compound 'D 860' in alloxan diabetic rats and dogs, respectively. They all observed that the sulphonamides have no effect on alloxan diabetes in animals.

Loubatieres (1944, 1944a, 1946) observed that IPTD (P-aminobenzene sulphamido isopropyl thidiazole) administration failed to influence blood sugar in complete pancreatectomized animals. In partial pancreatectomized (1/8 to 1/10 of pancreas left) animals, however, fall of blood sugar was observed after IPTD administration. This observation led Loubatieres to conclude that blood sugar lowering effect of IPTD depends on its stimulating effect on β cells to secrete insulin. La Barre and Reuse (*loc. cit.*) showed that IPTD was effective in lowering the blood sugar in animals rendered diabetic by small dose of alloxan (incomplete necrosis of β cells), but not in animals rendered diabetic by large dose of alloxan (complete necrosis of all β cells). This observation also supports the above view of Loubatieres.

Since an adequate dose of alloxan induces necrosis of β cells, keeping the structure and function of alpha cells intact, failure of Nadisan therapy in our experiment might be attributed to the absence of intact β cells (Plate VI, fig. 2) in alloxan diabetic rats. In other words, it can be presumed that Nadisan mediates its action through intact β cells of the island of Langerhans.

Our experiments further show that previous Nadisan feeding in animals cannot prevent the diabetogenic action of alloxan, this being probably due to inability of Nadisan to prevent the necrotic action of alloxan on the β cells of the pancreas.

SUMMARY.

1. Previous feeding of Nadisan to albino rats fails to prevent the diabetogenic action of alloxan.

2. Long continued feeding of Nadisan cannot cause any regression in alloxan induced diabetes of albino rats or rabbits. Nadisan therapy is equally ineffective, whether instituted early or late after the induction of the disease.

Authors' sincere thanks are due to the Drugs Controller, Government of India, for making Nadisan available. It was obtained through the courtesy of Messrs. Neo-Pharma (Private) Ltd., Bombay. Thanks are also due to Messrs. S. Banerji, M. A. Hai, S. K. Dass and H. M. Chakravarti, for their technical assistance.

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AN EXPERIMENTAL STUDY OF THE EFFECT OF SOME DRUGS ON TEMPERATURE IN NORMAL AND PYRETIC RATS.

BY

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THE present paper is concerned with the study of the effect of some drugs on the body temperature of normal and febrile rats. During the study of the 2,3-disubstituted quinazolones for their hypnotic activity (Gujral, Kohli and Saxena, 1955), marked sweating was observed in the animals receiving the compounds. It was, therefore, considered worthwhile to investigate their effect on body temperature.

Two compounds, QZ-1 (2-methyl-3-phenyl-4-quinazolone) and QZ-2 (2-methyl-3-orthotolyl-4-quinazolone), were selected for this study because the former produced maximum visible sweating and the latter showed the maximum hypnotic activity. Phenobarbitone was included as a representative of the barbiturate group of hypnotics in order to assess the temperature lowering component attributable to the effect of hypnosis *per se*. The three hypnotics were used in equi-effective doses ND_{50} , that is, the doses of the drugs required to induce narcosis in 50 per cent animals (Gujral, Kohli and Saxena, 1956).

For a comparative evaluation of the hypothermic and antipyretic activity of these compounds, a number of drugs affecting the normal and/or the fever temperature were included in the study, namely, chlorpromazine hydrochloride, acetyl salicylic acid, ACTH, cortisone acetate and desoxycorticosterone acetate. The last two drugs were used separately as well as in combination. Table I shows the drugs with their dose and route of administration employed.

TABLE I.

Drug.			Dose mg./kg.	Route of administration.
QZ-1	...	...	59	Oral
QZ-2	...	...	29	Oral
Phenobarbitone	...	...	50	Oral
Chlorpromazine	...	...	2	Subcutaneous
Acetyl salicylic acid	...	...	400	Oral
Cortisone	...	...	20	Oral
Desoxycorticosterone	...	...	5	Intramuscular
Cortisone + desoxycorticosterone	...	...	20, 5	Oral, intramuscular
ACTH	...	...	10	Intramuscular

METHOD AND MATERIAL.

Healthy albino rats between 150 g. and 200 g. in weight were used. They were divided in groups of eight, each group was used for testing one drug. The normal rectal temperature and its hourly variation over a period of 4 hours was recorded in eight groups, at the beginning of the experiment. The mean normal temperature was found to be 99.70°F . with a standard deviation of 1.21°F . (10 a.m., room temperature 68°F .). The average temperature change in these animals over a period of four hours was found to be $-0.6 \pm 3.72^{\circ}\text{F}$. (10 a.m. to 2 p.m., room temperature 68° to 72°F .). These observations indicate a wide variation of normal temperature in different rats and in the same rat at different times.

Pyrexia was produced by injecting subcutaneously a 15 per cent suspension of dried Brewer's yeast in 2 per cent acacia in normal saline. The injections were given at 6 p.m. and the volume injected was 1 c.c. of the suspension/100 g. of body-weight. About 15 hours later the rectal temperature of each rat was taken and found to be fairly stabilized. The mean rise of temperature in eight groups was found to be 1.41°F . with a standard deviation of 0.57°F . The temperature change in an untreated group over a period of four hours was found to be $0.70 \pm 0.88^{\circ}\text{F}$.

Chlorpromazine hydrochloride, ACTH and desoxycorticosterone acetate were employed in solutions and administered parenterally and the other drugs were suspended in 2 per cent gum acacia in distilled water and fed into the stomach through a cannula fitted to a syringe (Table I).

OBSERVATIONS AND RESULTS.

The observations were started immediately after administration of the drugs. They were recorded by the method used earlier in this Laboratory (Gujral *et al.*, 1955). The temperature lowering activity was assessed in terms of *mean* of the sum of the differences of hourly temperature from the initial temperature (the fever temperature in the case of pyretic rats) observed over a period of four hours. In the case of ACTH given to pyretic group of rats, it was found that the maximum effect was obtained during the first two hours when it was greater than that of

acetyl salicylic acid over the same period of observation. The antipyretic effect of ACTH mentioned in this study is, therefore, an underestimation of the true intensity of its action. Table II summarizes the results of the two sets of the observations. A rise of temperature over the initial temperature is indicated by + placed before the figure.

TABLE II.

Showing the hypothermic and antipyretic effect of the drugs in terms of the mean of the sum of the differences of the hourly temperatures from the initial temperatures in °F. observed over a period of four hours. Plus indicates a rise of the temperature over the initial temperature. Time. 9-30 a.m. to 2-00 p.m. Room temperature 68° to 72°F.

	APYRETIC RATS:		PYRETIC RATS:	
	Initial temperature.	Mean of the sum of the differences.	Initial temperature.	Mean of the sum of the differences.
Control	99.70	0.60± 3.72	101.18	+0.70± 0.57
QZ-1	100.55	> 19.75	101.00	13.35
QZ-2	100.15	11.60	100.75	2.35
Phenobarbitone	99.83	6.50	101.35	2.38
Chlorpromazine	99.60	9.91	101.20	3.80
Acetyl salicylic acid	100.63	5.50	101.18	11.70
ACTH	99.30	2.45	101.35	7.53
Cortisone	99.80	1.00	100.75	1.93
Desoxycorticosterone	98.70	+0.31	101.58	0.75
Cortisone + desoxycorticosterone	99.90	0.65	101.83	2.43

DISCUSSION.

The hypothermic effect.—A perusal of Table II shows QZ-1, QZ-2 and chlorpromazine to have a marked hypothermic effect. Considering the wide variation in the normal temperature over a period of four hours ($-0.6 \pm 3.72^\circ\text{F.}$), the effect of phenobarbitone and acetyl salicylic acid on normal temperature appeared to be slight. ACTH and the adrenal cortical hormones caused no significant change in temperature in the normal rats.

The antipyretic effect.—QZ-1 and acetyl salicylic acid produced a marked fall in fever temperature (Table II). Next in order of potency was ACTH. Chlorpromazine, combination of cortisone and desoxycorticosterone, phenobarbitone and QZ-2 produced only a slight fall and cortisone and desoxycorticosterone a negligible fall in fever temperature.

On the basis of their effects the drugs can be classified under two groups—those having a greater antipyretic effect and those having a greater hypothermic effect. It was observed (Table II) that acetyl salicylic acid and ACTH had a greater antipyretic than hypothermic effect. A similar tendency to lower the fever temperature more than the normal temperature was seen when a combination

of cortisone and desoxycorticosterone was used and to a lesser extent when these hormones were used separately. The effect of ACTH was more marked than the effect of the adrenal cortical hormones used in combination or separately. The different routes of administration employed with the two drugs (Table I) or an inequality in doses might be a contributory factor to this difference. The antipyretic effect of ACTH was quick in onset and of shorter duration when compared with that of acetyl salicylic acid. It was maximum during the first two hours and at this time it was greater than that of acetyl salicylic acid.

The observation that ACTH and adrenal cortical hormones lower the body temperature only in fever is in agreement with the observations of Seyle (1949) and Atkins *et al.* (1955) who suggested the suppression of the tissue reaction causing rise of temperature to be the probable mode of their antipyretic action. A similar mechanism of action is partly responsible for the observed greater antipyretic effect of acetyl salicylic acid through its capacity of stimulating the secretion of adrenal cortical hormones (Naach *et al.*, 1956).

The fact that the hypothermic effect of QZ-1, QZ-2 and chlorpromazine was greater than their antipyretic effect shows that the mechanism by which these drugs act might be different from that of acetyl salicylic acid, ACTH and the corticoids. The same may hold true for phenobarbitone which resembled the former drugs in the pattern of its action (Table II). The temperature lowering effect does not appear to be related to the hypnotic effect as QZ-1 lowered the temperature to a greater extent than QZ-2 and phenobarbitone although as far as their hypnotic effect is concerned, the three drugs were employed in equi-effective doses. The possible mechanisms by which chlorpromazine facilitates the reduction in body temperature have been described by several investigators (Courvoisier *et al.*, 1953; Dobkin *et al.*, 1954; Dundee, 1954; Decourt, 1953; Decourt, Gastel and Grenat, 1954). The 2, 3-disubstituted quinazolones have not been fully studied but on the basis of reports they appear to differ from chlorpromazine in that they are more potent hypnotic (Gujral, Saxena and Khanna, 1956) and are devoid of analgesic effect (Gujral, Kohli and Saxena, *loc. cit.*). It appears that the apparent similarity in their hypothermic and antipyretic effect to that of chlorpromazine may only partly be due to some similarity in the mechanism of their action.

The present study shows that QZ-1 possesses a powerful temperature lowering effect which is more marked on the normal than on fever temperature. The type of effect is similar to that of chlorpromazine and it will be interesting to investigate if QZ-1 can be employed with greater safety than chlorpromazine for producing hypothermia for therapeutic purposes.

SUMMARY.

The effect of two 2, 3-disubstituted quinazolones, 2-methyl-3-phenyl-4-quinazolone (QZ-1) and 2-methyl-3-orthotolyl-4-quinazolone (QZ-2) on the rectal temperature of normal and febrile rats has been studied and comparatively evaluated with that of phenobarbitone, chlorpromazine, acetyl salicylic acid, ACTH, cortisone, desoxycorticosterone and combination of cortisone and desoxycorticosterone.

The quinazolones showed a greater hypothermic than antipyretic effect and their temperature lowering effect appeared to be not related to their hypnotic effect.

The effect of QZ-1 and QZ-2 on body temperature in normal and fevered rats resembled that of chlorpromazine hydrochloride. An investigation of QZ-1, the more potent of the two quinazolones, with a view to its possible use for producing therapeutic hypothermia has been suggested.

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OCCURRENCE OF GLUCOSE IN COMBINATION WITH ACETO-ACETATE IN NORMAL BLOOD.

BY

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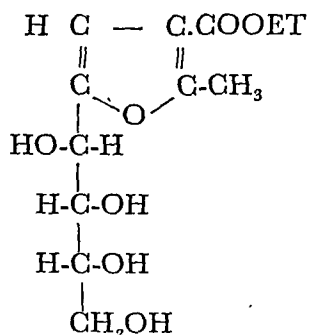
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GLUCOSE has long been known to occur in the body also in combination with other substances, such as proteins (Best and Taylor, 1950) and lipids (Hawk, Oser and Summerson, 1947). West (1927) succeeded in condensing glucose with ethyl aceto-acetate to give rise to a product whose structural formula has been shown by Gonzalez and Aparicio (1946) to be 2-tetrahydroxy butyl 5-methyl 4-carbethoxy furan (I).



(I)

It was postulated by Nath (1947) that the gradual accumulation of ketone bodies might be responsible for the onset of hyperglycemia and the rise in blood sugar might be caused not only through its overproduction or non-utilization but

also as a result of physiological response to combine, in a natural course, with some toxic metabolites in the system. Best and Taylor (1950a) have also supported this hypothesis and have shown how such combination may be considered as a protective mechanism against the excess accumulation of fat metabolites in the animal system.

Nath and Sahu (1956) observed that glucose reacts specifically with aceto-acetate alone and not with β -hydroxybutyrate, acetone or acetate, suggesting the existence of a definite metabolic relationship between glucose and aceto-acetate in the living system. This hypothesis can further be substantiated by other findings from this Laboratory (Nath and Sahu, 1954; Nath and Bhattathiry, 1956).

Having thus obtained some indirect proof of a metabolic relationship between glucose and aceto-acetate, we looked for some direct indication of an *in vivo* combination of these compounds in normal animals and this forms the subject of the present paper. Nath and Sahu (1954) have succeeded in showing the presence of glucose in combination with aceto-acetate in normal urine.

METHODS AND RESULTS.

(A) It was found that the condensation product on acid hydrolysis and subsequent neutralization with NaHCO_3 gave a colour reaction with ninhydrin at the room temperature similar to that reported by West and Rinehart (1947) for dienol compounds. Blood, when de-proteinized with trichloro-acetic acid and hydrolyzed with 2 N HCl, was also observed to give similar colour with ninhydrin and NaHCO_3 . It was, therefore, reasonably assumed that the normal blood also contains some such compounds which, on hydrolysis, might give rise to some dienol compound or compounds which are responsible for the development of such a colour with ninhydrin. That the condensation product of glucose and aceto-acetate, which can also be called as glucose-cyclo-aceto-acetate, on hydrolysis gives a dienol compound different from ascorbic acid, has recently been shown in this Laboratory through a special paper-chromatographic technique (Nath and Bhattathiry, 1956a) and it is through this technique that they could demonstrate the presence of a dienol glucose in the normal blood.

Now because normal blood also contains ascorbic acid, which is a dienol compound, it is quite possible that its presence will also interfere in this reaction and increase the intensity of colour. Attempts were, therefore, made at the outset to see if blood on hydrolysis is found to show greater intensity of such colour with ninhydrin than with non-hydrolysed blood in order to ascertain further whether a new dienol compound is liberated from the sample.

(B) 2.5 c.c. of blood samples obtained from brachial veins of young normal men (laboratory workers), marginal ear veins of male rabbits and sacrificed rats (all under non-fasting conditions) were de-proteinized with an equal volume of 10 per cent trichloro-acetic acid solution and filtered. The filtrate was then divided into 2 equal parts. 0.5 c.c. of concentrated HCl was added to one part and an equal volume of distilled water to the other which served as a blank. These portions were then heated for nearly 15 minutes in separate test-tubes kept immersed in boiling water. After the reaction was over, the portion containing acid was neutralized with NaHCO_3 . Ninhydrin and NaHCO_3 were added to both the

samples at room temperature (27°C.). The intensity of colour development was much more pronounced in the acid hydrolysed sample than that observed in the unhydrolysed blank, thus indicating the possibility of the dicnol compound other than ascorbic acid which rather gets partially destroyed during such hydrolysis.

Further, every sample of blood either from men or animals when de-proteinized and subjected to acid hydrolysis was found to give an increased value of reducing sugar and the samples after de-proteinization with H_2SO_4 and sodium tungstate, de-saccharification with $CuSO_4$ reagent and acid hydrolysis gave increased acetone values. The reducing sugar was estimated according to the method of Hagedorn and Jensen (1923) and acetone by Behre and Benedict's (1926) method. The results are given in Table I:—

TABLE

Free and combined glucose and aceto-acetate in normal blood of men and rabbits.*

Source of blood.	REDUCING SUGAR MG./100 C.C. OF BLOOD:			ACETO-ACETATE ESTIMATED AS ACETONE MG./100 C.C. OF BLOOD:		
	Before hydrolysis.	After hydrolysis.	Amt. liberated.	Before hydrolysis.	After hydrolysis.	Amt. liberated.
Men ...	109±1.9	185±2.8	76	0.45±0.08	1.25±0.08	0.8
Rabbits ...	107±3.4	180±3.1	73	2.5 ±0.33	5.5 ±0.45	3.0
Rats ...	95±1.5	155±2.1	60	0.9 ±1.9	2.5 ±0.25	1.6

* Each value is the average of 5 samples of normal blood.

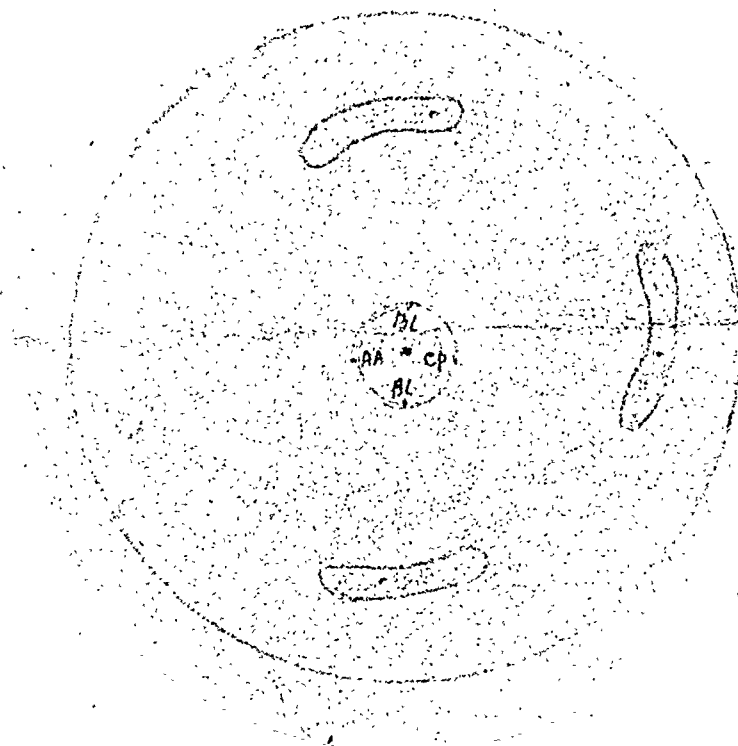
It was also noticed that a much greater increase in acetone value could be obtained if the acid hydrolysis was carried out at higher normalities of HCl. But such acid concentrations cannot be used for determination of glucose liberated as it gets decomposed under such conditions. That β -hydroxybutyric acid which might occur in the sample did not split off any acetone during the above acid hydrolysis, was also seen by carrying out actual acetone estimations with known amounts of β -hydroxybutyrate (Na salt) hydrolysed at different normalities of acid (HCl).

Next attempt was to see if some direct indication for the occurrence of glucose combined with aceto-acetate could be available by some chromatographic technique.

(C) Nath and Sahu (1954a) reported that the condensation product (Na salt) gives an orange red colour with 10 per cent $FeCl_3$ solution. This is quite distinct from the slightly deep brown colour given by aceto-acetate. Glucose does not show any colour with this reagent. This colour reaction was positive with deproteinized blood. The deproteinized blood sample was concentrated on the water bath, neutralized with Na HCO_3 and used for spotting the paper. An aqueous solution of the Na salt of the condensation product of glucose and aceto-acetate as well as aceto-acetate solution were used in the same chromatogram to find their comparative resolution. The circular paper chromatographic method

of Giri and Rao (*loc. cit.*) was used, with the solvent mixture of butanol : acetic acid : water (40:10:50 by volume). The chromatogram was developed by a spray of 10 per cent FeCl_3 solution and dried at room temperature, and the R_f values determined. The results are shown in the Figure.

FIGURE.



Showing the circular paper chromatogram of the (1) condensation product of glucose and aceto-acetate (CP) and (2) blood deproteinized (B1).
The solvent used is butanol : acetic acid : water (40:10:50) and the developing reagent is a 10 per cent ferric chloride solution.

DISCUSSION.

A careful study of the figure will indicate that the R_f value of the condensation product is 0.64 which is more or less the same with blood samples, i.e. 0.64 and 0.65, respectively, thus showing the presence of the condensation product of glucose and aceto-acetate in the normal blood, the indication of which was available from the results in the Table.

The present work will not only afford a definite clue about the source of the diol glucose in normal blood but will also establish at the same time, the *in vivo* combination of glucose with aceto-acetate in the normal system, postulated by Nath (*loc. cit.*) about ten years ago.

SUMMARY.

1. Hydrolysis of de-proteinized blood has been found to liberate a reducing sugar and aceto-acetate similar to that observed with the condensation product of glucose and aceto-acetate.

2. The presence of this condensation product has been identified in the normal blood by a chromatographic technique.

3. The Rf value of the condensation product in such chromatogram has been found to be 0.64.

4. A clue regarding the source of 1:2 diol glucose in normal blood has been suggested.

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ANTIBIOTIC PRINCIPLE FROM *MORINGA PTERYGOSPERMA*.

Part IX.

INHIBITION OF TRANSAMINASE BY ISOTHIOCYANATES.

BY

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THE possible correlation of the antimicrobial activity of pterygospermin with inhibition of action of the enzyme, transaminase, catalysing the system glutamic acid $\xrightarrow{\quad}$ alanine by benzylisothiocyanate (B.I.T.), one of the fission products of pterygospermin, has now been investigated along with two other representative isothiocyanates, viz., isobutyl (I.I.T.) and allyl (A.I.T.) isothiocyanates, which are known to occur in nature.

EXPERIMENTAL.

One per cent alcoholic solutions of isothiocyanates (Das *et al.*, 1957) were diluted to required concentrations with Sorenson phosphate buffer (M/15), pH 7.0.

The readily available green gram enzyme (Giri *et al.*, 1952) was used. Composition of reaction mixtures and the measurement of transaminase activity have been previously described (Kurup *et al.*, 1954).

RESULTS.

The effect of varying concentration of B.I.T., I.I.T. and A.I.T. on the system, glutamic acid + pyruvic acid $\xrightarrow{\quad}$ alanine + α -ketoglutaric acid, is given in Table.

TABLE

A. Effect of B.I.T., I.I.T. and A.I.T. on the system glutamic acid + pyruvic acid $\rightarrow$ alanine + α -ketoglutaric acid.

Substance.	Conc. μ g./c.c. (micromoles).	PERCENTAGE INHIBITION AFTER:		
		2 hours.	4 hours.	6 hours.
B.I.T.	6.25 (0.042)	35.7	16.7	4.1
	12.5 (0.084)	48.4	24.2	10.2
	25.0 (0.168)	60.3	43.5	30.6
	50.0 (0.336)	74.6	56.1	40.8
I.I.T.	12.5 (0.109)	7.7	1.9	1.3
	25.0 (0.218)	22.0	2.0	1.3
	50.0 (0.435)	38.5	11.6	5.1
	100.0 (0.870)	44.0	17.4	10.1
A.I.T.	25.0 (0.252)	32.0	15.5	10.8
	50.0 (0.505)	39.1	27.6	17.3
	100.0 (1.01)	54.7	37.9	32.6

B. Effect of B.I.T. on the system

Alanine + α -ketoglutaric acid $\rightarrow$ glutamic acid + pyruvic acid.

Conc. μ g./c.c. (micromoles).	PERCENTAGE INHIBITION AFTER:		
	2 hours.	4 hours.	6 hours.
6.25 (0.042)	41.8	23.1	12.1
12.5 (0.084)	55.4	30.2	16.8
25.0 (0.168)	67.6	51.5	37.7
50.0 (0.336)	80.3	61.6	46.8

The degree of inhibition by the three isothiocyanates rises with the increase in their concentrations but falls off with the duration of incubation.

DISCUSSION.

The three isothiocyanates, viz., B.I.T., I.I.T. and A.I.T. inhibit the activity of the green gram transaminase catalysing the reaction glutamic acid + pyruvic acid $\rightarrow$ alanine + α -ketoglutaric acid in varying degrees, but B.I.T. is more potent in inhibiting this as well as the reverse system, viz., alanine + α -ketoglutaric acid $\rightarrow$ glutamic acid + pyruvic acid. Moreover, the extent of inhibition which falls off gradually with time increases with the increase in concentration of isothiocyanates which may be due to their slow inactivation. Further, there appears to be a rough correspondence between the activity of isothiocyanates in this respect with their anti-staphylococcal activity (Das *et al.*, 1957). These results are thus not at variance with the view previously put forth as to the activity of pterygospermin and its probable *in vivo* breakdown to benzyl-isothiocyanate (Das *et al.*, 1954).

SUMMARY.

The effect of benzyl, isobutyl and allyl-isothiocyanates on the transaminase catalysing the reaction glutamic acid + pyruvic acid $\rightleftharpoons$ alanine + α -ketoglutaric acid has been investigated. All the three isothiocyanates inhibit the enzyme in varying degrees, but B.I.T. is more potent in inhibiting this as well as the reverse system. In all cases the extent of inhibition increases with the increase in concentration of isothiocyanates and falls off gradually with time. There appears to be a rough correspondence between the inhibition of transaminase by isothiocyanates and their antistaphylococcal activity. These results are not at variance with the view that the activity pterygospermin may be due to its *in vivo* breakdown to benzyl-isothiocyanate.

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STUDIES ON THE OPTIMUM LEVEL OF FAT IN THE DIET*.

BY

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THERE seems to be no exact knowledge at present on the amount of fat which will be required in the human diet. The recommended daily allowances of the National Research Council of U.S.A. placed the desirable fat level in the diet at 20 per cent or 25 per cent of the total calorie intake. For very active adults, this may be placed at 30 to 35 per cent of the total calories (*Nutr. Revs.*, 1950). The Nutrition Advisory Committee of the Indian Council of Medical Research is silent as regards the fat requirement of Indians and only recommends that liberal amounts of fat should be included in the diet, since fat provides more than twice the energy obtainable from carbohydrate or protein and fats act as vehicles for fat-soluble vitamins. Johnson and Kark (1947) showed that among American soldiers at hard work free choice of food resulted in 33 to 40 per cent total calories being derived from fat. But it is a moot point whether appetite and self-selection of food can be used as a guide to determine the desirable proportion of a food component in the diet.

Earlier work on the subject has been mainly devoted to the study of the comparative merits of animal fats and vegetable fats at different levels and the criterion used to assess the performance has chiefly been the growth of the albino rat.

Considerations, such as the carbohydrate component of the diet or the age of the animal have been reported to influence the findings and there appears to be

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no agreed conclusions on this point (Smith, 1948). While extensive data have been made available by such studies, the main point at issue, viz., the optimum level of fat in the diet still remains unsolved.

Considerations other than growth, such as nitrogen retention, efficiency of food utilization, reproduction performance etc., have also been employed to assess the value of different levels of fat (Forbes *et al.*, 1946; Scheer *et al.*, 1947, 1947a, 1947b). There appear to be two distinct facets in such works: Firstly, the effect of the complete absence of fat from the diet as against its presence in varying amounts, and secondly, the effect of varying levels of fat in the diet. Several of these studies have served to emphasize the salutary effects of the presence of a minimum amount of fat in the diet. It may be of interest in this connection to state that Scheer *et al.* (1947c) found high levels of fat to be beneficial in endurance tests with rats which were allowed to swim to exhaustion.

The other side of the picture also deserves some mention. The support given of late to an association, if not a well-established correlation, between larger intakes of fat and increased proneness to develop atherosclerosis or degenerative cardiovascular disease weights the scale in favour of lower and lower levels of fat in the diet (Keys, 1953). Administration of high fat diets has also been reported to result in a type of anæmia (Johnson *et al.*, 1943).

One of the chief drawbacks in these investigations appears to be the lack of any agreed criterion in judging the results. The growth of the animal as shown by gain in live weight can be assumed to be sum total of all effects on the animal and thus used to assess the results. This has been already adopted by several workers. However, this method has its own limitations, as for example, the gain in live weight may not all be due to new tissues laid down but may be due to larger fluid retention (*cf.* Deuel *et al.*, 1947).

Most of the workers in this field have carried out experiments with fats, such as margarine, corn oil, butter etc., and these fats are not very common in India. Even butter or ghee is consumed only by a negligibly small proportion of the population in India. Especially, in view of the earlier researches which have shown that in the rat at least, fats may have varying influences in nutrition and that the amounts needed in the diet for optimum growth may depend to a considerable extent upon the source of fat (*Nutr. Revs.*, *loc. cit.*), the need arises to investigate the problem with fats which are common in India.

The present paper presents the results of an attempt in this direction and in the absence of any other generally accepted criterion of assessment, gain in live weight of the young albino rat fed varying levels of butter, sesame and ground-nut oils in isocaloric regimens has been studied.

Of late, Elvehjem and his school of workers have adopted lowered liver fat accumulation in animals as a test of the nutritional adequacy of the protein and amino-acid component in the diet (Elvehjem, 1956). The influence of fat level also on liver fat was considered worthy of investigation and the present paper reports certain preliminary studies on the liver lipid status in animals continually fed varying level of fat in the diet.

EXPERIMENTAL.

The study consisted of three experiments. The variation in the three experiments was only with regard to the source of fat, viz., butter-fat, sesame or ground-nut oils.

The plan of each experiment was as follows: Weanling young albino rats were divided into five groups of eight animals each taking care to have an even distribution of sex, litter-mates and initial body-weight in the various groups. The first four groups were fed 2, 5, 10 and 20 per cent fat in the diet. These levels were chosen after due consideration of the range of fat levels in the Indian diets as revealed by the various dietary surveys. The rather high level of 20 per cent fat was also included to find out the possible effect of a high fat-level on growth. Casein, corn starch salts and vitamins formed the other components of the diet. The actual composition of the diets fashioned after the one employed by Barki *et al.* (1950) is given in Table I. The proportion of the calories contributed by the protein to the total calorific value of the diet was kept constant in all the four groups. The diets were fed, as far as possible, in isocaloric amounts to all the groups. The diets were renewed every week and kept in cold storage when not required so as to avoid development of rancidity.

TABLE I.

Composition of the experimental diets.

Dietary components, per cent.	GROUP NUMBER:			
	1	2	3	4
Fat* ...	2.0	5.0	10.0	20.0
Cane sugar ...	12.6	12.0	11.0	9.0
Corn starch ...	59.2	55.4	50.0	39.1
Vitaminized starch† ...	4.0	4.7	5.0	5.6
Casein‡ ...	18.2	18.9	20.0	22.3
Salt mixture ...	3.17	3.77	4.0	4.46
Calories per 96 g. of energy-yielding food ...	394	409	434	484
Grammes protein per calorie from the above diets ...	0.0462	0.0462	0.0462	0.0461
Ratio for feeding isocaloric amounts ...	1.102	1.061	1.000	0.897

*The source of fat was butter, sesame oil or ground-nut oil in experiments I, II, and III. Due allowance was made for the moisture content of the butter-fat samples while preparing the diets.

†An alcoholic solution containing the following amounts of vitamins was mixed with 100 g. corn starch, dried below 40°C. and used: Thiamine 4 mg.; riboflavin 6 mg.; pyridoxin 6 mg.; calcium pantothenate 30 mg.; choline 3 g.; vitamin A acetate 11.2 mg.; α -tocopherol 44.8 mg.; vitamin D₂ 0.28 mg.; menadione (vitamin K) 4.2 mg.; niacin 12 mg.

‡Rendered fat-free by extraction for two three-hour periods with boiling alcohol.

Since there was no means of judging the optimum growth, the fifth control group was kept on the laboratory stock ration and the growth of this last group of animals was assumed to be optimum. The laboratory stock ration consisted of a mixture of unleavened bread (chapati), sprouted Bengal gram, cow's milk, meat,

TABLE III.

Growth per calorie intake by rats fed various levels of butter-fat or sesame oil or ground-nut oil.

or ground-nut oil.

Group number.	Level of fat, per cent.	Average calories consumed.	Average gain in weight in 8 weeks.	Gain in weight per calorie consumed.	Statistical interpretation.
I. Butter-fat:					
Male rats.					
1	2	2,136	139	0.06520 ± 0.001863	1:2 N.S.
2	5	2,142	144	0.06733 ± 0.002136	1:4 N.S.
3	10	2,128	141	0.06613 ± 0.001574	1:3 N.S.
4	20	2,114	138	0.06504 ± 0.000873	2:3 N.S.
					2:4 N.S.
Female rats.					
1	2	2,022	104	0.05140 ± 0.001899	3:4 N.S.
2	5	2,012	106	0.05257 ± 0.003498	1:2 N.S.
3	10	1,959	99	0.05048 ± 0.003850	1:3 N.S.
4	20	1,940	100	0.05155 ± 0.001599	1:4 N.S.
II. Sesame oil:					
Male rats.					
1	2	2,110	121	0.05728 ± 0.006545	1:2 N.S.
2	5	2,127	124	0.06049 ± 0.003616	1:3 N.S.
3	10	2,104	124	0.06105 ± 0.003510	1:4 N.S.
4	20	2,064	133	0.06413 ± 0.002783	2:3 N.S.
					2:4 N.S.
Female rats.					
1	2	2,036	96	0.04724 ± 0.005088	3:4 N.S.
2	5	2,031	96	0.04737 ± 0.004222	1:2 N.S.
3	10	2,028	101	0.04984 ± 0.004124	1:3 N.S.
4	20	2,022	99	0.04876 ± 0.005246	1:4 N.S.
III. Ground-nut oil:					
Male rats.					
1	2	2,097	124	0.05732 ± 0.002188	1:2 N.S.
2	5	2,086	116	0.05559 ± 0.003052	1:3 N.S.
3	10	2,083	122	0.05858 ± 0.001532	1:4 N.S.
4	20	2,078	123	0.05991 ± 0.004212	2:3 N.S.
					2:4 N.S.
Female rats.					
1	2	2,059	80	0.04536 ± 0.001851	3:4 N.S.
2	5	2,043	87	0.04245 ± 0.000809	1:2 S.
3	10	2,038	93	0.04563 ± 0.001873	1:3 N.S.
4	20	2,038	89	0.04368 ± 0.001825	1:4 N.S.

S.—Significant. N.S.—Not significant.

S.—Significant. N.S.—Not significant.

of essential fatty acids and as a vehicle for fat-soluble vitamins has to be borne in mind.

Since the growth response to the variation in the level of fat in an adequate diet was not marked, an examination was made of the total liver lipid content of the animals fed varying levels of sesame oil. At the end of the test-period of eight weeks, the livers were removed according to the procedure of Henry *et al.* (1953) and the lipid content determined (Hawk and Elvehjem, 1953). The data on the liver lipid status appear in Table IV:—

TABLE IV.

Liver lipid status of rats fed varying levels of sesame oil in the diet.

Group number.	Level of fat, per cent.	LIVER LIPID PER CENT (ON DRY-WEIGHT BASIS)			Significance test.
		Range.	Average	Standard deviation.	
Male rats.					
1	2	4.7—17.8	11.8	±5.433	1.3; 1.2
2	5	3.1—17.3	11.8	±6.393	1.4; 1.5
3	10	10.1—15.3	11.5	±2.536	2.3; 2.4
4	20	6.0—12.3	9.7	±2.972	2.5; 3.4
5	Stock ration	3.5—10.8	6.9	±3.537	3.5; 4.5;
					N.S.
					S.
					N.S.
Female rats.					
1	2	4.0—14.0	9.7	±4.246	1.2; 1.3
2	5	4.2—14.7	9.2	±5.098	1.4; 1.5
3	10	6.3—11.4	8.9	±2.161	2.3; 2.4
4	20	2.7—7.6	5.6	±2.258	2.5
5	Stock ration	3.1—11.2	6.1	±1.215	3.4
					3.5; 4.5
					N.S.

S — Significant.

N.S. — Not significant.

There was a wide variation in the lipid content of the livers of animals subjected to the same dietary regime itself, e.g., from 3.1 to 17.3 per cent on 5 per cent sesame oil diet. It was, however, found that taking an overall picture, the animals in the control group (stock ration) and the high-fat diet (20 per cent) possessed less liver lipid store (about seven per cent) than the animals on lower levels of fat (about ten per cent). Due to the wide variation in the values for individual groups, the statistical interpretation of the data has to be accepted with due reserve and caution.

However, this finding is of some importance. The liver lipid status has been affected only by varying the level of fat in the diet, other factors, such as supply of methionine, choline, etc., not coming into the picture. There appears to be no systematic study in the literature on the liver lipid store of animals fed adequate diets but varying in levels of fat only. Harper *et al.* (1954), however, observed that with a corn oil or butter-fat diet, increase in the level of fat from 5 per cent to 20 per cent resulted in a slight decrease in liver fat and concluded that fats did not accumulate progressively in the livers of rats which received increasing amounts

of dietary fat. This finding is in accord with the present observations. It may be of interest to pursue this line of investigation with a larger number of animals and with other fats and also to know the character of such lipid deposits, whether it is cholesterol or phospholipid, etc., to gain further insight into this problem.

SUMMARY.

1. Young weanling albino rats were fed for eight weeks isocaloric diets but with levels of fat varying from 2 per cent to 20 per cent. Butter-fat, sesame and ground-nut oils were used as the sources of fat in the three separate trials.

2. Growth of a control group of rats on laboratory stock ration was assumed to be optimal and at none of the levels tested, the growth of the animals on the experimental diets equalled that of the control group.

3. When the growth of the animals was compared as gain in weight per calorie intake, no striking difference was observed among the several levels of each of the fat examined.

4. Animals on 5 per cent ground-nut oil ration showed the poorest growth.

5. While the data are not conclusive, the liver lipid store of animals receiving higher levels of sesame oil was observed to be less than that of animals receiving lower levels.

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NUTRITION AND TUBERCULOSIS.

Part I.

AN EPIDEMIOLOGICAL STUDY.

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It has long been believed that malnutrition tends to affect human tuberculosis adversely. It has, however, not been possible to provide indisputable scientific proof for this view. Considerable epidemiological, experimental and clinical evidence pointing to the deleterious rôle of malnutrition in tuberculosis exists, but such evidences can, at best, be considered only circumstantial and suggestive. For example, it has been pointed out by several observers that tuberculosis mortality rates in different economic groups in a community tend to vary inversely with their economic levels (Ewart, 1923; Wolff, 1938). There are also numerous observations pointing to increase in tuberculosis mortality in various countries during times of war and famine (Lee, 1931; Faber, 1938; Rich, 1946; Stowman, 1946; Keys *et al.*, 1950); but all these evidences pointing to increase in tuberculosis mortality rates in times of privation need not necessarily be considered conclusive proof of the aggravating rôle of malnutrition in tuberculosis. During war and famine apart from malnutrition, there is also a simultaneous deterioration in environmental conditions. The possible contributory effects of such deterioration in environmental factors have also to be considered. However, even when due allowance is made for these factors a careful examination of the epidemiological evidence would appear to point to the aggravating rôle of malnutrition in tuberculosis. The subject has been exhaustively and ably reviewed in a number of publications (Rich, *loc. cit.*; Keys *et al.*, *loc. cit.*).

Malnutrition and tuberculosis are among the major public health problems of underdeveloped countries. It is important to consider how these two problems

which are both widespread and which happen to co-exist over vast regions of the globe tend to interact with each other. Whether the problem of tuberculosis is considered from the larger point of view of prevention and control of the disease, or from the narrower point of view of therapeutic management of individual cases, the question of the rôle of malnutrition in tuberculosis assumes great importance. To be able to plan a preventive campaign against tuberculosis in underdeveloped countries and to approach the problem of dietary management of tuberculosis cases rationally, it is necessary to have information on this subject.

THE PRESENT STUDY.

In the present study, the question of the probable rôle of malnutrition in tuberculosis was investigated on the following lines:

1. *Comparison of the incidence of tuberculin positivity in a group of malnourished children on the one hand and apparently normal children of the general population on the other.*—The incidence of tuberculin positivity in a group of 548 children aged up to 10 years and suffering from various clinical manifestations of malnutrition was compared with that in a group of apparently normal children of the same age composition and belonging to the general population. The clinical manifestations encountered among the malnourished group is indicated in Table I. Using 0.1 c.c. (10 tuberculin units) of P.P.D., tuberculin tests were performed on all the subjects. Precautions were taken to exclude cases that had received previous B.C.G. vaccination, and those children who were suffering from conditions known to cause a false negative reaction. The responses were read 48 hours following the tests by measuring the diameter of induration over the injected area. In line with the criterion employed by the B.C.G. teams, an induration exceeding 5 mm. in diameter was considered positive. To assess the reliability of tuberculin positivity in malnourished children, x-rays of the chest were taken in 29 malnourished cases returning positive reaction chosen at random.

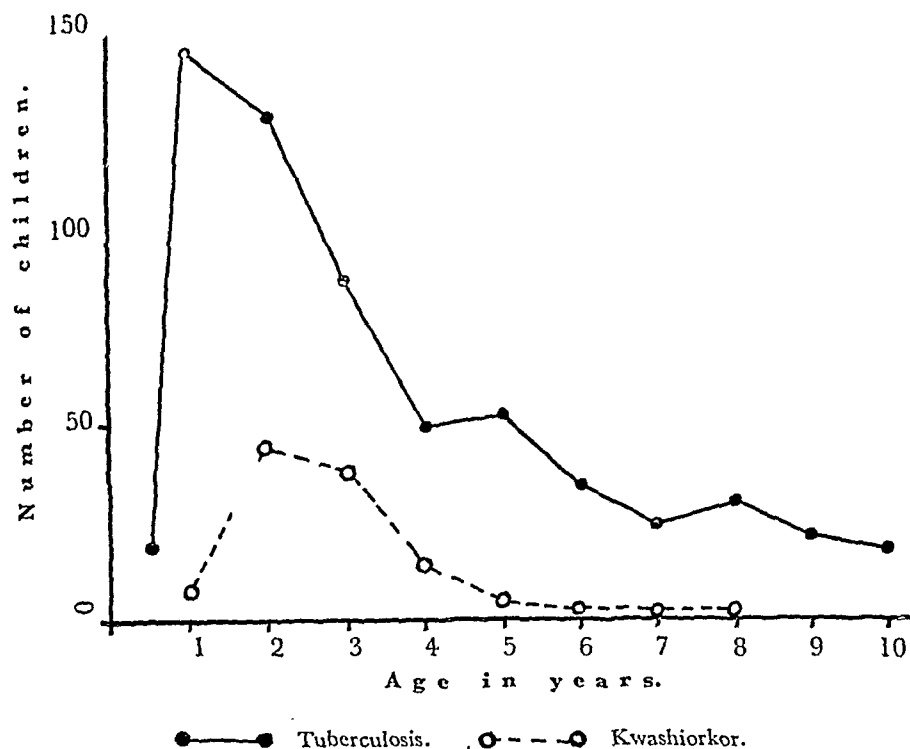
TABLE I.

Clinical types of cases of malnutrition studied.

Clinical type.	Number of cases.
Vitamin A deficiency	328
Protein malnutrition	54
Protein malnutrition + vitamin A deficiency	47
Vitamin B complex deficiency	11
Protein malnutrition + vitamin B-complex deficiency	11
Vitamin B-complex deficiency + vitamin A deficiency	15
Simple undernutrition	82

To investigate the possibility that malnutrition might contribute to false negative tuberculin reaction in children of the undernourished group, 20 undernourished children who returned negative tuberculin response were administered a daily supplement of 30 g. of protein in the form of skim milk for a period of 1 to 2 months and the tuberculin tests were repeated in those cases at the end of this period.

GRAPH.



Incidence of tuberculosis and kwashiorkor in children of different ages.

2. *Comparison of the incidence of tuberculosis and kwashiorkor among children of the same socio-economic group.*—Information regarding the incidence of tuberculosis in cases of kwashiorkor in different regions was gathered. The respective age distribution of cases of tuberculosis and kwashiorkor among children of the same socio-economic group was studied.

RESULTS.

The incidence of tuberculin positivity among a group of malnourished children and a group of apparently normal children of the general population has been indicated in Table II. It will be noticed that in the age group under six years there was no significant difference with regard to the incidence of tuberculin positivity as between the malnourished and the control subjects. In the age group between 6 and 10 years, the incidence of tuberculin positivity was actually greater in the control group than in the malnourished group. Of the 29 children from the malnourished group with positive reaction who were chosen at random, x-rays of the chest revealed radiological evidences suggestive of tuberculosis in 22 cases.

Of the 20 children with initial negative response who were subsequently treated with high protein diets, 3 returned a positive response on retesting.

TABLE II.

Incidence of tuberculin positivity among malnourished children and children of general population.

Age.	CASES OF MALNUTRITION:			General population, per cent.
	All cases of malnutrition, per cent.	Cases of vitamin A deficiency, per cent.	Cases other than vitamin A deficiency, per cent.	
0-6 years ...	11.8	14.1	6.7	11.4
6-10 years	18.3	18.7	16.7	32.8

An attempt to gather information on the incidence of tuberculosis in cases of kwashiorkor was made in the expectation that this may throw some light on the question of the rôle of protein malnutrition in tuberculosis. Unfortunately, however, it appeared that not all clinicians dealing with kwashiorkor had examined their cases carefully for evidence of tuberculosis. The information that was collected was somewhat contradictory. Trowell (1956) in Kampala who had Mantoux-tested and x-rayed all his cases of kwashiorkor, reported that the incidence of tuberculosis in his cases was between 2 and 5 per cent. On the other hand, according to the same author, nearly one-third to one-half of kwashiorkor cases in Johannesburg in South Africa were seen to suffer from tuberculosis. Harish Chandra (1956) in Hyderabad reported that out of 186 cases of kwashiorkor investigated by him in 1955, 59 cases had radiographic evidence of pulmonary tuberculosis and 3 others had tuberculosis of the cervical glands—an incidence as high as reported by Trowell in South Africa. Pierce (1956) in Mysore, on the other hand, found there was no significant difference with regard to the incidence of positive Mantoux-reaction as between a group of kwashiorkor cases and normal controls. In a group of 25 cases of kwashiorkor studied in the course of the present investigation, radiological evidences suggestive of tuberculosis was obtained in 9 cases, though none of these was clinically active.

Information regarding the age distribution of cases of tuberculosis (of all categories) and of kwashiorkor admitted under Achar in 1954 in the Government General Hospital, Madras, was also gathered. The results are indicated in the Graph. The peak incidence of tuberculosis seemed to precede that of kwashiorkor, but the period between 1 and 3 years in which kwashiorkor was the major problem was also the period of maximal incidence of childhood tuberculosis.

DISCUSSION.

The results of the comparative study of the incidence of tuberculin positivity in malnourished and normal children have to be interpreted with considerable caution. This approach may fail to reveal the aggravating rôle of malnutrition

in tuberculosis for two reasons: (1) malnutrition may be contributing to false negative Mantoux-reaction, and (2) several cases of malnutrition affected with tuberculosis may have failed to survive with the result that the malnourished group investigated may be a selected group from which cases of tuberculosis had already been weeded out. That malnutrition could contribute to false negative tuberculin reaction has been shown by several workers (Debre and Papp, 1926; *Faber, loc. cit.*; Wolff, 1930; Mitchell *et al.*, 1928; Wilson and Miles, *loc. cit.*). The limited study of the effect of nutritional rehabilitation on the initial negative tuberculin response obtained in 20 cases in the present investigation would seem to support this view. The appropriate conclusion that may be drawn from the results obtained here is *not* that malnutrition has no effect on tuberculosis but that tuberculin tests in malnourished populations may provide an unreliable indication of the incidence of tuberculosis. In comparing the results of tuberculin positivity in malnourished and well-nourished subjects, the possible effects of variations in environment in the two groups on the incidence of tuberculosis must also be considered. In the present investigations, however, both the malnourished and the apparently normal group were drawn from the same environment.

The reasons for the wide divergence in the reported incidence of tuberculosis in different groups of kwashiorkor cases are not clear. Trowell (*loc. cit.*) found the incidence of tuberculosis as low as 5 per cent in his cases of kwashiorkor in Uganda, but considered the incidence of tuberculosis among cases of kwashiorkor in Johannesburg to be much higher. One finding which may be of significance in this connection is that the incidence of vitamin A deficiency signs among kwashiorkor cases, as judged from all available reports, is apparently much higher in South Africa than in Kampala. Experiments on rats carried out in these laboratories (Sriramachari and Gopalan, 1957) indicated that vitamin A deficiency may diminish resistance to tuberculosis, an observation which is in line with that reported by Finkelstein (1932).

The observations of Pierce (*loc. cit.*) in Mysore are again contradictory to those of Harish Chandra (*loc. cit.*) in Hyderabad. It must be pointed out, however, that the former author had depended on the Mantoux-reaction for the assessment of the incidence of tuberculosis. The reliability of Mantoux-test, however, in cases of kwashiorkor is questionable. In support of this, it was observed in the course of recent investigation that in eleven cases of advanced malnutrition showing clinical and radiological evidences of tuberculosis, the Mantoux-reaction was negative. This would again illustrate the point that in advanced malnutrition false negative tuberculin reactions are probably very frequent. Further intensive investigations of the incidence of tuberculosis in malnutritional states may provide important information bearing on the problem of the rôle of nutrition in tuberculosis.

SUMMARY.

1. The incidence of tuberculin positivity in a group of 548 malnourished children aged up to 10 years has been compared with the incidence of tuberculin positivity in a group of apparently normal children of the general population.

2. The relative incidence of tuberculosis and kwashiorkor in children of the same socio-economic groups has been studied.

3. The significance of these observations has been discussed.

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NUTRITION AND TUBERCULOSIS.

Part II.

STUDIES ON NITROGEN, CALCIUM AND PHOSPHORUS METABOLISM IN TUBERCULOSIS.

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IN an effort to elucidate the interrelationship between nutrition and tuberculosis, some workers have tried to investigate the metabolism of different nutrients in patients suffering from tuberculosis (McCann, 1922; Johnston, 1953; Brewer *et al.*, 1950 and 1954; Co Tui *et al.*, 1954). While such studies cannot obviously yield information on the rôle of malnutrition in predisposing to tuberculous infection, they may be expected to provide some clue regarding the nutritional requirements in tuberculosis and the possible effect of undernutrition on the course of established tuberculosis cases.

It may be expected that the metabolism of different nutrients in tuberculosis is influenced by the severity of the infection and possibly also by the preceding nutritional status of the subjects with regard to the nutrients concerned. The fact that these two factors cannot always be truly assessed in human cases of tuberculosis, imposes an important limitation on the interpretation of the results of metabolic studies carried out on human cases. In experimental animals, however, these factors can be controlled; but the use of experimental animals for metabolic studies raises the question of the applicability of the results obtained in such investigations to human subjects. The problem would, therefore, seem to need an approach from both the clinical and experimental angles.

In the present investigation, the metabolism of nitrogen, calcium and phosphorus has been investigated in human cases of tuberculosis and in guinea-pigs in whom tuberculosis had been induced.

Case No. 5.—Male, aged 35 years, agricultural labourer. History—cough with expectoration since 4 years, haemoptysis off and on since 2 years, sweating since one month. Had had 30 g. streptomycin + P.A.S. once 3 years ago. Clinical condition—weight 106 lb., x-ray lungs—big tension cavity with infiltration right apex, heart—normal, urine and faeces—*nil* abnormal, E.S.R.—50 mm./1 hour. R.B.C.—3·6 million. Hb.—10 g. per cent. Total W.B.C.—6,600 per cmm. Sputum—positive for T.B.

Case No. 6.—Male, aged 18 years, agricultural labourer. History—fever, cough, sweating, loss of weight and pain in the left side of the chest since 1 to 1½ months. Clinical condition—weight 94 lb. Temperature—101·2°F. Pulse—110 per minute, x-ray lungs—right lung infiltration apex and intraclavicular region, left lung normal, heart normal, urine and faeces, *nil* abnormal, E.S.R.—55 mm./1 hour. R.B.C.—3·6 million, Hb.—10 g. per cent. Total W.B.C.—5,000 per cmm. Sputum—positive for T.B.

Case No. 7.—Male, aged 42 years, domestic servant. History—cough with expectoration since 5 years. Clinical condition—weight 100 lb., x-ray lungs—right lung infiltration apex and intraclavicular region, left lung—normal. E.S.R.—3 mm./1 hour. R.B.C.—4·5 million. Hb.—17 g. per cent. Total W.B.C.—6,200 per cmm. Sputum—positive for T.B.

Case No. 8.—Female, aged 22 years. History—fever off and on since one year, loss of body weight since a year, pain in the left side of the chest, cough with expectoration since one month. Clinical condition—weight 54 lb., x-ray lungs—infiltration left apex and intraclavicular region. right lung—normal. Heart—normal. Urine and faeces—*nil* abnormal. E.S.R.—35 mm./per hour. R.B.C.—4·19 million, Hb.—12 g. per cent. Total W.B.C.—10,400 per cmm. Sputum—positive for T.B. Plasma proteins—5·7 g. per cent. Albumin—2·6 g. per cent.

Cases 1 to 7 were immediately placed on a standard diet, the composition of which is indicated in Table II. There was some minor difference between the composition of the standard diet given to cases 1 to 6 on the one hand and the cases 7 and 8 on the other, as shown in the table. Case No. 7 and case No. 8 (in the initial period) were maintained on the same diets which they were used to consume at home. The patients were maintained on the respective diets for 7 days before metabolism studies were undertaken on them. The diets were carefully weighed and it was ensured that the patients consumed all the food supplied to them. After the 7-day adjustment period, metabolism studies were carried out for three-day periods in all cases. Urine and faeces passed during the study were carefully and completely collected. Sputum was collected in two cases and nitrogen estimated by micro-Kjeldahl procedure. Since nitrogen loss through sputum during 24 hours did not exceed 100 mg., loss of nitrogen through sputum was considered not significant. Nitrogen, calcium and phosphorus were estimated in urine by the methods mentioned above in the case of guinea pigs. Determination of nitrogen, calcium and phosphorus were carried out in faeces as described by Stearns (1929). In patients 1 to 7, the metabolic studies were carried out on only one occasion as above.

In Case No. 8, however, four serial metabolic studies—two before treatment and two after treatment were carried out. After the completion of the first

metabolic study, this patient was placed on a different diet supplying a higher level of protein (104 g. daily as against 76 g. daily provided by her first diet). After allowing another seven days for adjustment to the new diet, a metabolic study similar to the first was carried out for a three-day period. The patient was then treated with calcium P.A.S., isoniazid and streptomycin. During this period the patient was on the latter diet supplying 104 g. protein daily. After 17 days of treatment during which period the patient was beginning to improve clinically, the metabolic study was repeated for a three-day period. The treatment was continued for another 68 days at the end of which appreciable improvement in the clinical conditions of the patient had occurred. At this stage, the last metabolic study was carried out again for a three-day period.

TABLE II.

Composition of the diets of tuberculosis patients.

Particulars.	Patient 1 to 6.	Patient 7.	PATIENT 8:	
			During the first period (I metabolic study).	During the second period (II, III and IV metabolic study).
Protein ...	105	87	76	104
Fat ...	81	38	38	42
Calories ...	3,284	2,781	2,327	2,576

RESULTS.

The results of the experimental study on guinea-pigs are indicated in Table III. The results of clinical study on human cases are set out in Tables IV and V.

It can be seen from Table III that guinea-pigs, before infection, retained considerable amount of nitrogen, calcium and phosphorus on both high protein and low protein diets. The total quantity of the nutrients retained was much higher in the high protein group than that in the low protein group. Per cent retention of nitrogen and calcium was slightly lower in low protein group as compared to the high protein group, whereas the per cent retention of phosphorus was identical in both the groups. The differences in the per cent retention of nitrogen in the two groups, which is statistically significant ($t=3.02$), might be due to (i) differences in the level of protein in the diet, and (ii) differences in the quality of protein present in the two diets (Table I).

After infection there was a marked reduction in the quantities of nitrogen, calcium and phosphorus retained in both the groups (Table III) which was out of proportion to the small reduction in the intake of these nutrients. This will be evident when the per cent retention on the two diets is considered. A common feature in the metabolism after infection was that the faecal as well as the urinary excretion of the three nutrients was increased in both the groups indicating a defect both in absorption and utilization. An important difference between the animals on the high protein diet and those on the low protein diet was noticed with

TABLE III.
Nitrogen, calcium and phosphorus metabolism in guinea-pigs before and after infection.

Group.	Number of animals.	Average wt. (g) change during metabolism.	AVERAGE NITROGEN IN G.				AVERAGE CALCIUM IN G.				AVERAGE PHOSPHORUS IN G.						
			Intake.	Faecal excretion.	Urinary excretion.	Balance.	Per cent retention.	Intake.	Faecal excretion.	Urinary excretion.	Balance.	Per cent retention.	Intake.	Faecal excretion.	Urinary excretion.	Balance.	Per cent retention.
High protein Before infection	11	+17	2.3590	0.3461	0.2401	+1.7728	75.15	1.0037	0.2313	0.0133	+0.7591	75.60	0.5747	0.2195	0.0150	+0.3402	59.20
High protein After infection	6	-18.3	1.9362	0.5904	0.6950	+0.6508	33.61	0.5210	0.3943	0.0442	+0.0825	15.80	0.4165	0.3859	0.0674	-0.0368	...
Low protein Before infection	11	+22.80	6.4390	1.6710	0.0576	+0.4192	65.10	0.4338	0.1190	0.0184	+0.2964	68.30	0.3287	0.1139	0.0175	+0.1973	60.00
Low protein After infection	5	-10.00	5.6560	0.4455	0.2935	-0.1734	...	0.1839	0.0838	0.0521	+0.0480	26.10	0.1998	0.1422	0.0495	+0.0081	4.50
Low protein After infection-- fed high protein diet	3	-4.8	1.2824	0.7094	0.6442	-0.0712	...	0.3450	0.3016	0.1020	-0.0586	...	0.2758	0.3908	0.1108	-0.2258	...

TABLE IV.
Nitrogen, calcium and phosphorus metabolism in tuberculosis patients.

Patient number.	Weight change during 10 day period, kg.	NITROGEN IN G.				CALCIUM IN G.				PHOSPHORUS IN G.			
		Intake.	Faecal excretion.	Urinary excretion.	Balance.	Intake.	Faecal excretion.	Urinary excretion.	Balance.	Intake.	Faecal excretion.	Urinary excretion.	Balance.
1	+1.82	16.44	4.83	7.72	+3.59	1.47	1.64	0.15	-0.32	2.42	1.29	0.94	+0.19
2	+0.91	16.44	2.44	7.12	+6.88	1.47	0.79	0.15	+0.53	2.42	0.64	0.88	+0.90
3	0	16.44	1.69	6.63	+8.12	1.47	0.60	0.04	+0.83	2.42	0.32	0.88	+1.22
4	+1.82	16.71	1.68	7.84	+7.19	1.28	0.62	0.08	+0.58	2.42	0.46	0.82	+1.14
5	+0.68	17.03	2.18	6.12	+8.73	1.48	0.48	0.05	+0.95	2.48	0.53	0.59	+1.36
6	0	17.03	3.14	7.36	+6.53	1.48	0.78	0.15	+0.55	2.48	0.97	0.85	+0.66
7	+0.68	13.98	2.64	9.80	+1.54	1.66	1.51	0.15	0	...	...	...	...

Figures represent average of three 24-hour collections.

regard to the nitrogen balance after infection. All the animals of the high protein group maintained positive balance, though the per cent retention was very much lower than that before infection. On the other hand, most of the animals of the low protein group showed negative nitrogen balance. With respect to calcium and phosphorus retention, the pattern was the same in both the groups; both positive and negative balances being observed in the two groups.

TABLE V.

Serial nitrogen metabolism study in patient 8.

Period.	NITROGEN IN G. PER DAY.				
	Intake.	Faecal excretion.	Urinary excretion.	Total excretion.	Balance.
<i>Before treatment:</i>					
3rd-6th day after admission ...	12.19	3.01	3.36	6.37	+5.82
13th-16th day after admission	16.63	4.21	4.30	8.51	+8.12
<i>After treatment:</i>					
33rd-36th day after admission	16.63	3.75	6.24	9.99	+6.64
101th-103rd day after admission	16.63	4.30	3.27	7.57	+9.06

When the animals of the low protein group were switched over to a high protein dietary regime for 7 days they still showed negative balance of nitrogen. This indicates that the decreased retention of these nutrients in these animals after infection cannot be corrected by a temporary increase of protein intake for a short period, after infection had set in. Prolonged high protein feeding before and after infection may make a difference. This fits in with the observations of other workers that negative nitrogen balance that sets in after a stress-like infection (Grossman *et al.*, 1945) and surgical operation (Cuthbertson, 1946; Koop *et al.*, 1946; Howard *et al.*, 1944) cannot be corrected by temporarily increased nitrogen intake.

There was no correlation between weight changes, on the one hand, and retention of nitrogen, calcium and phosphorus after infection on the other. The animals of the high protein group, though maintaining positive nitrogen balance were not gaining weight.

Results of metabolic study with human cases of tuberculosis indicated that there was considerable retention of nitrogen, calcium and phosphorus (Table IV). The retention observed in these cases seemed very much higher than in normal subjects (*cf.* McKay *et al.*, 1942; Mitchell, 1949; Patwardhan *et al.*, 1949). This type of high retention had been observed by several other workers in tuberculous patients (Johnston, *loc. cit.*; Co Tui *et al.*, *loc. cit.*), in other infectious diseases (Grossman *et al.*, *loc. cit.*; Waife *et al.*, 1950) and in undernutrition (Bray, 1953; Holmes *et al.*, 1954; Venkatachalam *et al.*, 1955). The extent of retention observed in these cases varied from patient to patient. This was especially striking in the case of nitrogen retention, being 1.5 g. per day, on the one hand, in patient No. 7 and about 9 g. per day, on the other, in patients No. 5 and No. 8. There was parallelism between nitrogen retention, on the one hand, and calcium and phos-

phorus retention, on the other. Only one patient (No. 1) showed negative calcium balance; this is attributable to high calcium excretion in stools which was bulky. Similarly, large quantities of nitrogen and phosphorus were excreted in faeces of this patient compared to other patients. It was found that the degree of retention of nitrogen could not be correlated with blood sedimentation rate, temperature, weight or age of the patients. However, patient No. 7 who had normal E.S.R. (3 mm./hour) showed minimum retention.

The results of the serial metabolic studies with patient No. 8 have been set out in Table V. Metabolic studies were conducted on this patient on two levels of protein (76 g. and 104 g.) before treatment and twice afterwards on 104 g. protein. The patient retained considerable quantity of nitrogen on 76 g. of protein which was her usual diet. But when the protein intake was raised to 104 g. nitrogen retention also increased considerably. The patient continued to retain nitrogen nearly to the same degree as in the beginning even after 3 months of treatment when there was clinical improvement in her condition.

It is also seen from Table V that there was no correlation between nitrogen retention and weight changes. There was maximum weight increase in patient No. 1 with minimum nitrogen retention (excluding patient No. 7), while there was no change in weight in patient No. 3 and weight loss in patient No. 8 with maximum retention. This is an observation reported by several workers, not only in tuberculosis (Johnston, *loc. cit.*; Co Tui *et al.*, *loc. cit.*; McGann, *loc. cit.*), but in other infections (Waife *et al.*, *loc. cit.*; Sappington of Bockus, 1949; White and Spriggs, 1901; Johnson, 1946), and in undernutrition (Bray, *loc. cit.*; Holmes *et al.*, *loc. cit.*; Venkatachalam *et al.*, *loc. cit.*).

DISCUSSION.

The foregoing results indicate that the metabolism of nitrogen, calcium and phosphorus in guinea-pigs on diets of high and low protein content before infection are such as would be expected of normal growing animals. The differences in the two groups as to the retention of these nutrients reflect the differences in protein level, and the protein quality of the two diets. However, after infection there was a marked change in the metabolism of these nutrients, there being greater excretion and less retention on intakes similar to those before infection. Between the two groups of animals there was a marked difference after infection as regards protein metabolism. The animals of high protein group were in positive balance though the total quantity retained was much less than that before infection, while most of the animals of low protein group showed negative balance.

It is a well established fact that protein metabolism is profoundly influenced in states of stress, such as infection, surgical operation, trauma, burns, etc. (Pollack and Halpern, 1951; Peters and Van Slyke, 1946). For example, within a few days of surgical operation and burns large quantities of nitrogen are lost in the urine (Howard *et al.*, *loc. cit.*; Cuthbertson, *loc. cit.*; Croft and Peters, 1945). The degree and rapidity of this nitrogen loss is influenced by the proceeding nutritional status of the subject, a well nourished subject tending to get into negative nitrogen balance more readily than an ill-nourished subject (Browne *et al.*, 1945; Browne, 1948; Cuthbertson *loc. cit.*). This phase of protein metabolism, the 'catabolic

phase' cannot be corrected by temporary high protein feeding (Koop *et al.*, *loc. cit.*; Howard *et al.*, *loc. cit.*; Cuthbertson *loc. cit.*).

In the light of this observation it may be concluded that the animals of high protein group must have passed through an acute catabolic phase immediately after the infection which from the available evidence was probably characterized by an even more pronounced negative nitrogen balance than that of animals of the low protein group.

The present investigation, however, shows that the catabolic phase was reversed very much earlier in the animals on the high protein group than in those on the low protein group, with the result that when the metabolic studies were repeated after infection, the animals of the high protein group were already in positive nitrogen balance, while those of the low protein group were still in negative balance. It could be concluded from this study that high protein intake before and during infection is helpful in tiding the animal more rapidly through the phase of negative nitrogen balance, which ensues immediately after an infection like tuberculosis. An inadequate protein intake, on the other hand, might prolong the catabolic phase and chances of survival during this critical period might be expected to be correspondingly reduced.

In human cases of tuberculosis, it was evident that on intakes of 100 g. of protein per day (or even 70 g./day) large quantities of nitrogen were retained. This would indicate that all these patients were in the anabolic phase where the protein stores are repleted, depletion having occurred in the catabolic phase immediately after infection. This avidity for protein may indicate the extent of tissue protein depletion in these cases.

One interesting finding was that the fluctuations in the body-weight occurring in cases of tuberculosis may sometimes provide a misleading indication of N balance of the individuals. There was no correlation between weight changes and nitrogen retention. This was specially well illustrated by the behaviour of patient No. 8 (Table V). This patient was retaining large quantities of nitrogen throughout, but showed both weight loss and weight gain. At the time when her clinical conditions had improved (after three months) there was no net change in her weight.

Another important point that arises from the present metabolic studies on human cases of tuberculosis pertains to the protein requirement in tuberculosis. In deciding the optimal requirement of protein in tuberculosis the mere presence of positive nitrogen balance as in normal adults cannot be a satisfactory criterion. The protein intake resulting in maximal retention must be considered the optimal requirement. Even a small positive balance in these subjects may not indicate that all the tissues of the body are in positive balance (Allison, 1955). These patients having great avidity for protein may show N balance at relatively low intake (McCann, *loc. cit.*) which cannot be taken as their optimal requirement. This approach to the question of protein requirement in tuberculosis has been borne out by the observations on Case No. 8. It was observed that in this patient even 76 g. protein which the patient was accustomed to consume at home was sufficient to maintain her in positive nitrogen balance. However, when the protein intake was raised to 104 g. per day further retention could be induced in this patient.

Since metabolism studies were not carried out at levels higher than 104 g. protein per day, it has not been possible to decide the level at which maximal nitrogen retention is achieved. It can, however, be concluded that 100 g. of protein per day was nearer the optimal protein requirements of this subject than 76 g. protein. Phansalkar and Patwardhan (1956) had shown that the minimum protein intake required to maintain a normal Indian adult in N balance was of the order of 38 g. per day. It would, therefore, seem that in a case of tuberculosis of moderate severity the protein requirement is more than doubled. Browne and his group (quoted by Johnston, *loc. cit.*) had shown that though N equilibrium could be induced on 11 g. of nitrogen per day in a tuberculous patient a marked increased retention of nitrogen was observed by raising the nitrogen intake to 15 g. and 25 g. a day.

Another point in favour of high protein intake was illustrated in patient No. 8. Though the patient was receiving treatment before she was taken up for investigation her clinical condition was actually deteriorating. When she was placed on high protein diet and the same treatment continued, appreciable clinical improvement set in. The impression was gained that even the patients No. 1-6 improved in their general condition during the 10 days when they were on high protein diet though they were not receiving any specific treatment. Our conclusion regarding the need for high protein therapy in tuberculosis is borne out by the investigations of Johnston (*loc. cit.*), Co Tui *et al.* (*loc. cit.*) and Glogowski (1955). It was interesting to notice that even after 3 months on high protein diet, the patient No. 8 was still retaining considerable quantity of nitrogen. This would show the extent of tissue depletion in cases of tuberculosis and the need for maintaining high protein diet therapy for prolonged periods.

SUMMARY.

1. Nitrogen, calcium and phosphorus balance was determined in guinea-pigs with experimentally induced tuberculosis and in eight human cases of tuberculosis.

2. Animals on the high protein diet recovered from the negative balance phase following on infection more rapidly than those on the low protein diet.

3. All the human cases of tuberculosis showed large positive nitrogen, calcium and phosphorus balance. Body-weight changes sometimes offered misleading indication of the status of N retention. Positive nitrogen balance was found to be associated with good prognosis.

4. Using the criterion that the protein intake resulting in maximal N retention is the optimal protein requirement, cases of tuberculosis require not less than 100 g. of protein per day.

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NUTRITION AND TUBERCULOSIS.

Part III.

EFFECT OF SOME NUTRITIONAL FACTORS ON RESISTANCE TO TUBERCULOSIS.

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THE epidemiological evidence pointing to the possible rôle of malnutrition in tuberculosis has been briefly reviewed in an earlier paper (Jayalakshmi and Gopalan, 1957). Even if it is conceded that the epidemiological evidence does point to the rôle of malnutrition in tuberculosis, one has necessarily to turn to experimental observations to decide the specific nutrients involved. Unfortunately, much of the experimental evidence on this subject is also confusing and contradictory (Rich, 1946; Sengupta and Howe, 1949; Metcoff *et al.*, 1949). Considerable caution would seem to be necessary in the interpretation of the experimental data. The natural history and the evolution of tuberculosis in the experimental animal and the human being need not necessarily be the same; the nutritional requirements of the animal and the human subjects are also different. These considerations constitute important limitations to the experimental approach.

In the present investigation, an attempt has been made to study the possible rôle of (a) protein deficiency, (b) vitamin A deficiency, and (c) caloric deficiency, in influencing the susceptibility of guinea-pigs and albino rats to experimentally induced tuberculosis. The above two species of animals were deliberately chosen because one of them, the guinea-pig, is known to be highly susceptible to tuberculosis infection and the other, the albino rat, highly resistant. The investigation mainly concerned the effects of deficiencies of protein, vitamin A and calories for the reason that these constitute the major nutritional deficiencies among poor Indian communities. In interpreting the data obtained, due note has been taken of the limitations involved in an experimental study of this kind.

MATERIAL AND METHODS.

Guinea-pigs.—Twentyfour young male guinea-pigs weighing about 200 g. each were divided into two groups of twelve animals each. One group was maintained on a high protein diet and the other on a low protein diet. The composition of these diets have been indicated in Table I of an earlier paper (Narasinga Rao and Gopalan, 1957). The change-over from the colony diet of the animal (sprouted Bengal gram and cabbage) to the experimental diet was effected gradually so as to enable the animals to get accustomed to these diets. The diets were fed *ad lib.* After the animals had subsisted on the respective experimental diets for one month, during which period the animals were growing steadily, the animals were placed in an isolated animal house and were infected with tubercle bacilli. The infection was allowed to develop for twelve weeks at the end of which the animals were sacrificed. Post-mortem examination was conducted on these animals as well as on those which had died during the 12 week interval, and their various organs were examined histologically. Throughout the study, a careful serial record of the weights of the animals was maintained.

During this study, nitrogen, calcium and phosphorus metabolism in these animals were determined just before and after 12 weeks after infection. The results of this study have been reported separately (Narasinga Rao and Gopalan, *loc. cit.*).

Rats.—Young weanling rats weighing about 40 g. were divided into 3 groups of 33 animals each. The groups were identical with respect to sex, litter, and initial weight distribution. The three groups were maintained on a 2 per cent, 5 per cent and 18 per cent protein diet, respectively. The diets were fed *ad lib.* Eight weeks after the above groups were started, two more groups of young rats of 22 animals each were included. These two groups were respectively fed on (1) a 18 per cent protein diet without vitamin A, fed *ad lib.*, and (2) a 18 per cent protein diet restricted to 50 per cent of the calculated normal consumption. There were thus in all five groups of animals involved in the experiment. The details regarding the groups and their diets are indicated in Table I.

After the animals had been on their respective diets for some days (nine weeks in the case of groups 1, 2 and 3 and 3, days in the case of groups 4 and 5), the rats were placed in an isolated animal house and were infected with tubercle bacilli. Seven weeks after the infection, all the rats were sacrificed. Systematic post-mortem examination was conducted on these as well as those rats that had died after inoculation. Different organs of these rats were histologically examined. Throughout this study a careful serial record of the weekly weights of these animals was maintained.

Inoculation.—Guinea-pigs and rats were infected with a suspension of HRV 37 strain of tubercle bacilli in physiological saline. The strain was subcultured on glycerine-agar medium and the organisms gently scraped from the surface and carefully weighed; appropriate amount of physiological saline to give a concentration of 1 mg. of bacilli in 1 c.c. of saline was added and ground in a mortar to obtain a uniform suspension. In the case of the guinea-pigs, 0.1 mg. each of the bacilli (in 0.1 c.c. of the suspension) was injected subcutaneously in the region of

the symphysis pubis. Rats were infected through the intraperitoneal route with 1 mg. each of the bacilli (in 1 c.c. of the suspension).

TABLE I.

Composition of high and low protein diets of rats.

Components.	Two per cent protein diet (group I).	Five per cent protein diet (group II).	High protein diet (groups III, IV* and V†).
Amylum ...	88.2	84.1	64.2
Casein ...	2.8	6.9	26.7
Gingelly oil ...	5.0	5.0	5.0
Salt mixture (a) ...	4.0	4.0	4.0
Vitamin mixture (b) ...	0.1	0.1	0.1
Choline ...	0.1	0.1	0.1

(a)—Salt mixture of Briggs *et al.* (1952).

(b)—Vitamin mixture Heinicke *et al.* (1955).

*This group was not administered vitamin A; the diet was otherwise identical with that of group III.

†This group received half of the diet received by group III; the qualitative composition of the diet was otherwise identical with that of group III.

Groups IV and V were started on the experiment 8 weeks after the first three groups but animals of all groups were inoculated simultaneously.

Post mortem and histological examination.—All the animals were systematically examined after death. The severity and extent of involvement of the several abdominal and thoracic viscera were recorded and representative samples of all the organs were taken for histological examination. The tissues were fixed in formalin and were processed through paraffin. All the tissues were stained with hæmatoxylin and eosin, and the Ziehl-Nielsen technique. On the basis of morphological appearances, the lesions were arbitrarily graded as follows: (1) epithelioid (non-caseating) tubercles, (2) caseating tubercles, (3) tuberculous granulation tissue, (4) miliary tuberculosis, (5) fibro-caseous tuberculosis, (6) calcifying tubercles.

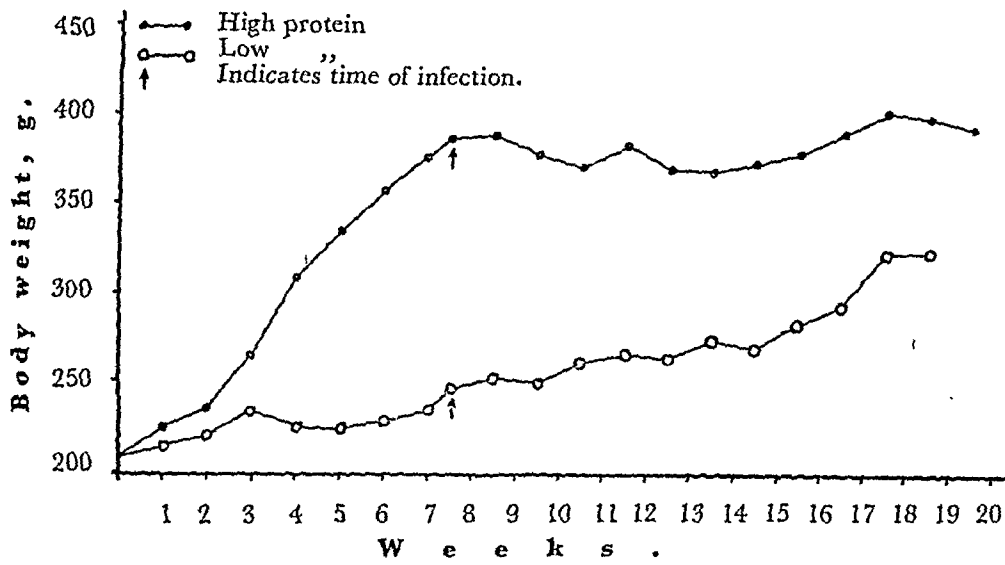
The severity in each type of lesion was assessed by the extent and the relative number of tubercle bacilli contained therein.

RESULTS.

Growth curve.—The growth curves of guinea-pigs and rats are represented in Graphs 1 and 2.

In the case of guinea-pigs (Graph 1), it was observed that the animals of the high protein group grew rapidly before infection, but after infection their body-weights remained practically constant. This lack of growth after infection may be the result of infection or the normal decrease in the growth potential of the animals. On the other hand, the animals of the low protein group which were already growing slowly before infection continued to maintain the same low trend of growth even after infection. In the case of rats (Graph 2), the animals of all the groups maintained nearly the same trend of growth before and after infection.

GRAPH 1.



Mortality.—Examination of the results presented in Table II would reveal that the level of protein in the diet had no significant influence on the mortality and survival time of the infected guinea-pigs. Similarly, it would appear that the level of proteins in the diet had no marked influence on the mortality and survival time of the infected rats also. It is no doubt true that 19 animals in the 2 per cent protein group had died within the experimental period following on infection, but this could hardly be deemed significant considering that the mortality in this group even prior to infection was also nearly as high. These observations point to the lack of influence of protein level in the diet on the *immediate* mortality of experimental animals within seven weeks after infection.

GRAPH 2.

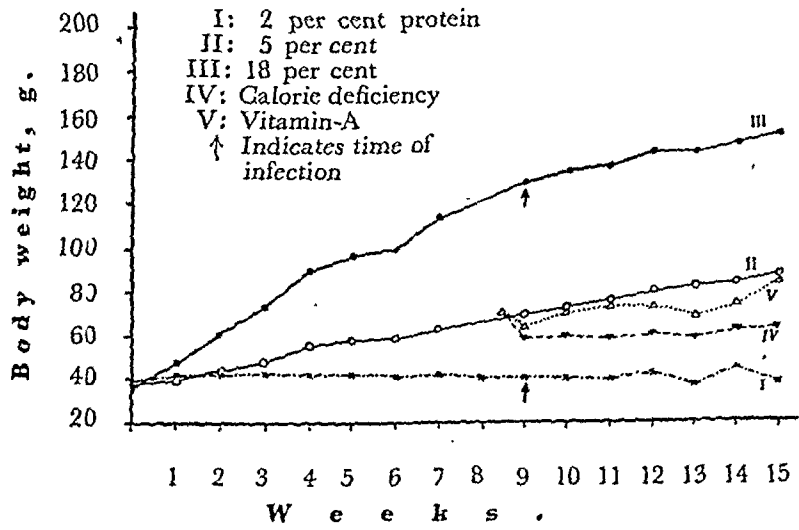


TABLE II.

Mortality and survival of experimental animals.

Experimental animal.	Diet.	Number of animals.	MORTALITY AND SURVIVAL TIME BEFORE INFECTION.		MORTALITY AND SURVIVAL TIME AFTER INFECTION.	
			Number of deaths.	Days after commencement of experiment when deaths occurred.	Number of deaths.	Days after infection when deaths occurred.
Guinea-pigs	High-protein ...	12	1	20	5	15, 42, 46, 63, 71
	Low-protein ...	12	2	43, 58	6	3, 42, 47, 61, 63, 72
	Two per cent protein	33	14	35, 40, 40, 44, 46, 46, 47, 48, 48, 57, 57, 57, 61, 62	19	2, 2, 2, 5, 9, 10, 11, 12, 13, 14, 16, 16, 17, 17, 23, 26, 27, 29, 47
Rats	Five per cent protein	33	Nil	...	1	25
	Eighteen per cent protein ...	33	1	41	Nil	...
	High protein 18 per cent + low caloric vitamin A deficient	22	Nil	...	10* 3†	15, 16, 17, 17, 18, 19, 21, 22, 25, 34 18, 29, 29

* and † = Mortality in a control group of 16 animals simultaneously on the same diets was 6 and nil, respectively.

Ten animals on the low-calorie diet (group 5) had died within the experimental period after infection; in the same period 6 of 16 animals maintained on the same diet without infection had died. Evidently, therefore, restriction of caloric intake to 50 per cent of the normal level did not appreciably influence the immediate mortality in the period of seven weeks following on infection.

Three animals on the vitamin A deficient group (group 4) had died within the experimental period after infection; in the same period none of the 16 animals maintained on the same diet without infection had died. This difference, however, is perhaps not significant.

HISTOLOGICAL EXAMINATION.

Guinea-pigs.—Animals from both groups showed varying degrees of tuberculous infection of the abdominal viscera with mildest lesions. Regional lymph nodes were found to be enlarged to the size of a split pea. The spleen was moderately enlarged. Other abdominal and thoracic viscera showed practically very little involvement. In the moderately severe cases, the lesions in the lymph nodes were somewhat more extensive and the abdominal viscera, notably the spleen and liver were found to be markedly enlarged and apparently caseating. Only

in a few animals, a serofibrinous peritoneal exudate was seen. Macroscopically the thoracic viscera were very little affected. A few tiny superficial tubercles were seen over the lungs in most of the animals.

Histological examination revealed that except for a few regional lymph glands which showed small caseating tubercles, all the other parenchymatous organs had extensive areas of tuberculous granulation tissue with practically no evidence of epithelioid tubercles, and caseation necrosis. The epithelioid and caseating tubercles in the lymph nodes and the granulation tissue contained very few tubercle bacilli. All these findings are in accordance with the primary type of lesions. Although the lungs showed no marked changes, there were small foci of tubercle granulation tissue with stray bacilli in almost all the animals examined.

Both macroscopic and microscopic examination revealed no differences in the character, distribution and severity of lesions (Plate VII, figs. 1 to 4) or the number of tubercle bacilli between the high and low protein-fed groups. This is illustrated in Table III. It can be observed from the table that with regard to susceptibility to infection, as well as to the severity there was no difference between the two groups.

TABLE III.

Summary of histological findings.

Experi- mental animal.	Diet.	Number of animals inoculated.	Number of animals infected.	DISTRIBUTION OF SEVERITY OF LESIONS:			
				Very mild.	Mild.	Moderate.	Severe.
Guinea- pigs	High protein diet ...	11	10	...	8	...	2
	Low protein diet	10	9	...	5	2	2
Rats	Two per cent protein diet ...	19	8	...	8	...	...
	Five per cent protein diet ...	33	21	6	7	8	...
	Eighteen per cent protein diet ...	32	19	2	12	5	...
	High protein (18 per cent) low calorie diet ...	22	10	3	7	...	...
	Vitamin A deficient diet ...	22	18	...	7	8	3

Rats.—The data regarding the incidence and the severity of infection are presented in Table III. Many animals in the 2 per cent protein group died early in the course of the experiment. The few that survived showed evidence of infection. About 60 to 65 per cent of the animals from all the groups except vitamin A deficient group were found infected whereas in vitamin A deficient group 81 per cent of the animals were found infected. But judging by the extent and severity of the lesions the animals receiving 2, 5 and 18 per cent protein diets showed about the same degree of involvement. The picture was similar in the group receiving high protein low calorie diet, whereas vitamin A deficient animals exhibited a relatively more severe infection. As is to be expected with primary

infection, the lesions were mainly confined to a few regional lymph nodes in the abdominal cavity. The spleen was generally enlarged (2 to 3 times the usual weight) although no macroscopic lesions were observed. Macroscopically the liver was practically normal. The diaphragm was found to have few tubercles. In all the animals the lungs were apparently normal. In the vitamin A deficient group the affected lymph nodes were larger and relatively more in number.

Histologically, there were no obvious differences in the character and severity of lesions between the animals of all the groups except the vitamin A deficient group. The affected lymph nodes contained both epithelioid and caseating tubercles. It was not possible to draw any correlation between the level of dietary protein and the presence and extent of caseating lesions (Plate VIII, figs. 5, 6 and 7). In vitamin A deficient group, however, the lymph nodes showed a more uniform picture with relatively large caseating tuberculous lymph nodes (Plate VIII, fig. 8). Unlike the lesions observed in the guinea-pigs these tuberculous nodes were filled with a large number of tubercle bacilli packed in the epithelioid cells and also in the form of large clumps in the centres of caseous masses. However, there did not appear to be any differences in the bacillary content of the tuberculous lesions of the several animals in a particular group or between the several groups. Even the vitamin A deficient animals showed a similar picture. The parenchymatous organs, especially the liver, lungs and spleen were involved to a mild degree compared with those of guinea-pigs. Extensive foci of tuberculous granulation tissue seen in the guinea-pigs were conspicuously absent in the case of rats. Microscopically, the spread of infection in the animals was evident as seen by the small and tiny collection of macrophages containing tubercle bacilli. These differences between the rats and guinea-pigs might reflect species differences in the response to infection. But as in the case of guinea-pigs there was no gross or histological differences between the group of rats receiving various levels of proteins or calorie deficient diet. Although the same type of lesions were observed in the vitamin A deficient group they were somewhat more extensive.

The results with rats also confirm the finding with guinea-pigs that the level of dietary protein has hardly any influence on the susceptibility to primary type of infection. The same conclusion emerges in the case of calorie deficiency. However, vitamin A deficiency seems to increase the susceptibility of rats to tuberculous infection.

DISCUSSION.

In the present study, it has been shown that the level of protein in the diet had no appreciable influence on the resistance of guinea-pigs and rats to tuberculous infection, or on the mortality within seven to twelve weeks after infection. It would, however, be unjustifiable to conclude from this that protein nutrition has no influence on the course of tuberculous infection. In the first place, these observations pertain to 'primary' infection and not to 'reinfection'. Secondly, these studies deal only with the immediate or short-term effects of infection. Nitrogen metabolism studies have shown that the level of protein in the diet has no influence on the extent of negative nitrogen balance which inevitably follows infection (Grossman *et al.*, 1945). During the period immediately following on infection,

therefore, marked differences between animals on high protein diet and those on low protein diet might not be expected. Nitrogen metabolism studies performed several weeks after infection had, however, shown that animals on the high protein diet tend to recover from the negative nitrogen balance phase immediately following on infection, much earlier than those on the low protein diet (Narasinga Rao, and Gopalan, *loc. cit.*). It might, therefore, be expected that the influence of the lack of protein in the diet would be more apparent in the long term studies.

In the present investigation, restriction of calorie intake to 50 per cent of the normal consumption did not significantly influence the resistance of rats to tuberculous infection or the mortality. These observations again pertain to the immediate effects of infection. Dubos (1955) had also observed that chronic starvation, unlike intermittent starvation, had no marked influence on the development of tuberculosis in mice. The influence of more drastic restriction of calories was not studied in the present investigation.

The histological studies indicated that among rats on the vitamin A deficient diet the incidence of infections was considerably higher and the lesions also more extensive than in the control group. It would appear from this that vitamin A may play some part in determining the resistance to tuberculous infection, a conclusion which is in line with the observation of Finkelstein (1932). The rôle of other vitamins remains to be elucidated.

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MODE OF ACTION VITAMIN D.
HEALING OF EXPERIMENTAL RICKETS BY STARVATION
AND ASSOCIATED BIOCHEMICAL CHANGES.

BY

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AND

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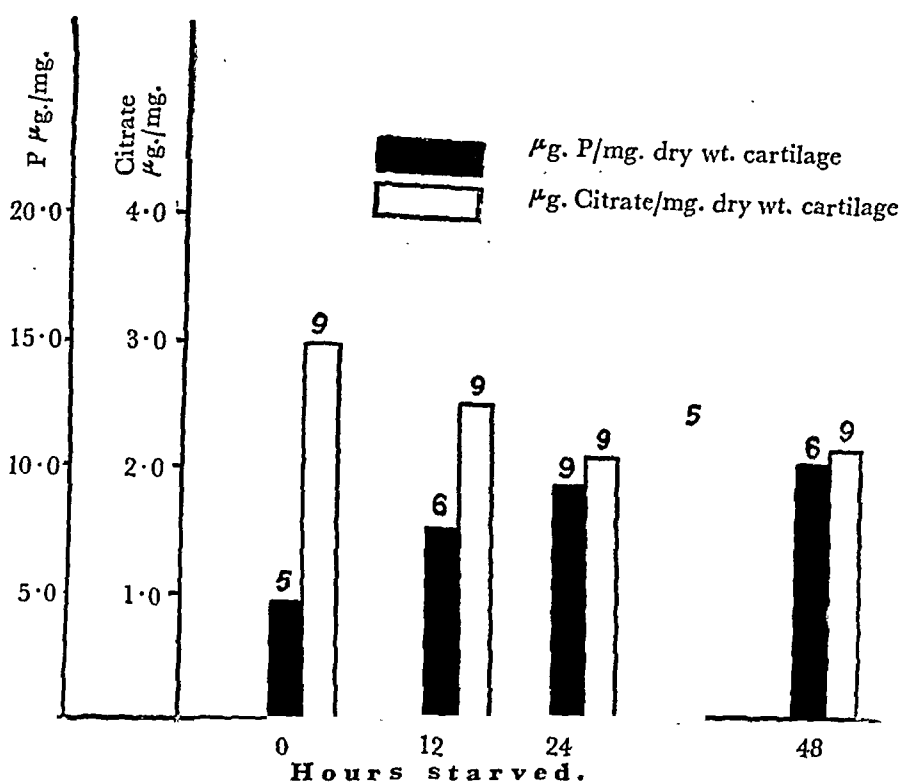
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McCOLLUM *et al.* (1922) observed the deposition of calcium salts in the epiphyseal cartilage of rachitic rats when the latter were starved for 3 to 5 days. They also reported that the process was identical with that observed when cod-liver oil was fed to rachitic rats. Both the above treatments brought about the formation of the zone of provisional calcification leading to the healing of rickets. Weinmann and Schour (1945) have reported a similar observation on the dentine of rachitic rats in whom calcification was resumed when the animals were starved for 48 hours or longer. The formation of new alveolar bone in the upper jaw of normal rats in whom the upper molar teeth had been removed was observed by Glickman (1945) when the rats were subjected to acute starvation. It appears, therefore, that starvation acts as a stimulus to calcification, even restoring the process where it has been interrupted as in induced rickets.

The fact that such diverse treatments as the administration of vitamin D, or of citrate (Shohl, 1937) or starvation alone bring about the healing of rickets is in itself of sufficient interest to deserve careful study. Ramalingaswami *et al.* (1954) have demonstrated by histochemical techniques that the changes taking place in the rachitic epiphyseal cartilage of the albino rat during healing caused by the administration of vitamin D or citrate were essentially similar. Sriramachari, *et al.* (to be published) have further observed that somewhat similar changes are initiated by starving rachitic rats for about 48 hours. It should be clear, therefore, that whatever be the agent which sets in motion the mechanism

GRAPH 1.



Effect of starvation on the total P and citrate content of rachitic rat cartilage.

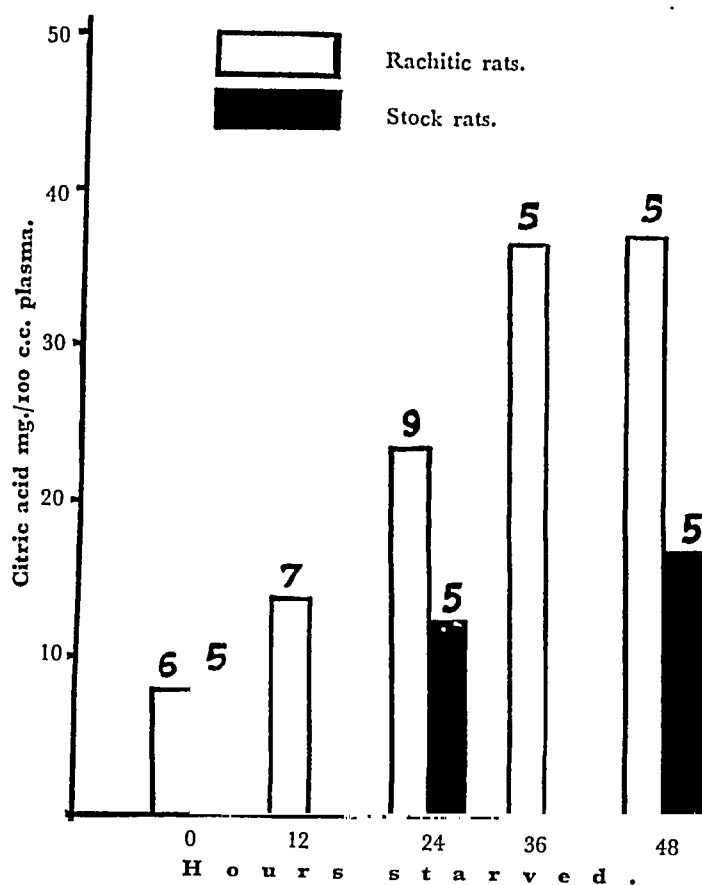
(Figures on top of the column indicate the number of animals.)

Influence of starvation on the plasma citric acid of rachitic and stock control rats.—The plasma citrate of the rachitic rats increased considerably during starvation as shown in Graph 2; the increase being over four-fold after 36 hours of fasting, the initial and final values being 8.24 ± 1.34 and 36.54 ± 5.36 mg. per 100 c.c. plasma, respectively. The plasma citrate of stock rats of the same age starved for similar periods also showed a significant rise, although not as sharp as was observed in the rachitic animals. Harrison (1953) and Joshi, Dikshit and Patwardhan (1957) have reported that the plasma citrate level is considerably lower in rickets and that it is elevated after vitamin D administration. Similar findings in rachitic rats subjected to starvation which are now reported are of great interest.

DISCUSSION.

The present investigation was undertaken with a view to studying the mechanism of healing of rickets induced by starvation and comparing the results with those obtained by the administration of vitamin D and citrate in rickets. It was felt that such a comparison would considerably improve our understanding of the process of calcification. As shown by Gutman and Gutman (1941) and Ramalingaswami *et al.* (*loc. cit.*) it would appear that the process of calcification is interfered with in rickets due to a disturbance in carbohydrate metabolism in the

GRAPH 2.



Influence of starvation on the plasma citric acid content of normal and rachitic rats.
(Figures on top of the column indicate the number of animals).

rachitic cartilage itself. That such a disturbance in fact occurs in vitamin D deficiency in rats was confirmed by the observations of Tulpule and Patwardhan (1954) who found that rachitic rat cartilage had a diminished capacity to oxidize pyruvate. The fact that the administration of citrate to rachitic rats had induced healing with histochemical changes in the epiphyseal cartilage similar to those observed on vitamin D administration led to the reasonable assumption that the disturbance in carbohydrate metabolism in vitamin D deficiency should be between the pyruvate and citrate stages. This was confirmed by Dikshit, Joshi and Patwardhan (*loc. cit.*) who observed a low citrogenase activity and a low citrate content in rachitic cartilage. The enzyme activity increased by more than 100 per cent even within 24 hours after administering vitamin D. Similar results were obtained in experiments with rachitic dogs (Joshi, Dikshit and Patwardhan, *loc. cit.*). These results lend strong support to the hypothesis that an arrest or slowing down of the formation of citrate in the epiphyseal cartilage is in some way responsible for the failure of calcification. As a corollary it should follow that

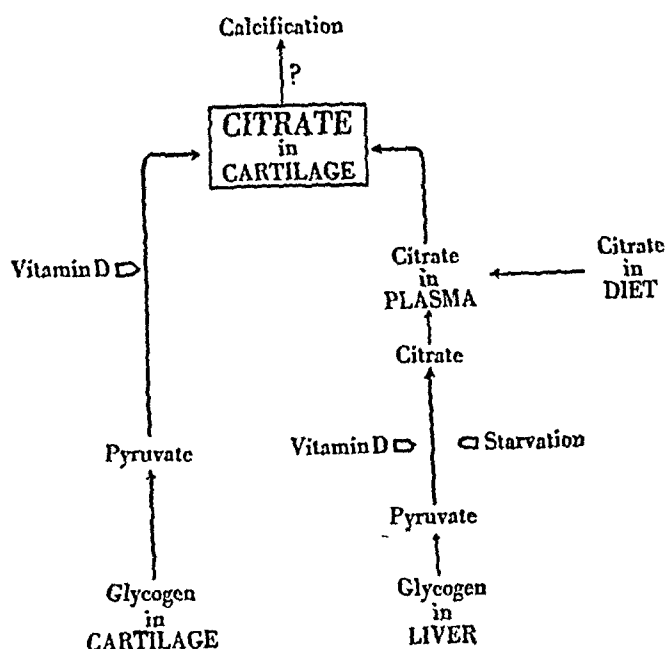
if citrate from extraneous sources could be made available in sufficient amounts to the epiphyseal cartilage, calcification would be resumed which, in fact, is what has been observed by Shohl (*loc. cit.*) and Ramalingaswami *et al.* (*loc. cit.*). On the other hand, in vitamin D treated animals, citrate becomes available at the site not only owing to its increased synthesis by the epiphyseal cartilage, but also from blood in which citrate level increases possibly on account of the increased citrate synthesis taking place in the liver under the influence of vitamin D (Joshi, Dikshit and Patwardhan, to be published.)

The results of starvation of rachitic rats described in the preceding pages show that the healing of the rachitic lesions was accompanied by a considerable rise in plasma citrate, although there was no rise in cartilage citrate. This last observation could be explained on the assumption that the rate of utilization of citrate brought to the site by the blood is so high as to prevent its accumulation in the cartilage. It must be admitted, however, that this assumption has to be subjected to experimental proof.

It has been suggested by Ramalingaswami *et al.* (*loc. cit.*) that citrate metabolism may be connected with the formation of the appropriate matrix (necessary for calcification), the quality of which appeared to be altered in rickets as demonstrated by histochemical methods. Although the present experiments do not indicate the mechanism of participation of citrate in the calcification process, they lend support to the concept that any procedure which would overcome the citrate block in carbohydrate metabolism should restore calcification impaired by a deficiency of vitamin D. It is thus possible to visualize a common pathway of the processes leading to the healing of the rachitic lesion brought about by such apparently unconnected treatments as vitamin D, citrate and starvation. This idea is illustrated in Text-figure 2.

The observations of Joshi, Dikshit and Patwardhan (*loc. cit.*) indicate that the action of vitamin D in promoting citrate synthesis is not restricted to the epiphyseal cartilage alone, but can be seen in liver as well. It is possible that it may extend to other tissues in a similar way although it has yet to be proved. Assuming that the effect is general and not specific to the calcifying cartilage the question naturally arises why a reduction in the rate of citrate formation specifically affects calcification. This question is difficult to answer although instances of an adverse demonstrable effect of a general disturbance in metabolism being limited to any one tissue or system are not unknown. For example, in the deficiency of vitamin B₁ there is a disturbance of carbohydrate metabolism resulting in the accumulation of pyruvate in the tissue fluids, and yet the effect of pyruvate increase is most marked on the brain cells resulting in the derangement of the nervous system (Peters, 1953). Then again, the signs of riboflavin deficiency are manifested primarily at the mucocutaneous junctions in the body, although the general concentration of this vitamin in its functional form as a flavoprotein may be decreased in tissues, such as liver, kidney, etc. (Snell, 1953). It is probable that the tissues specifically affected in this manner are more sensitive than others to a particular metabolic defect. In view of the above considerations the concept of inter-relationship between calcification and citrate metabolism as postulated in the present communication would appear to fall in line with the phenomena observed in thiamine and riboflavin deficiencies.

TEXT-FIGURE 2.



Suggested common pathway of the action of vitamin D, citrate and starvation in the healing of rachitic cartilage.

SUMMARY.

1. Young albino rats were fed a rachitogenic diet for 21 days and then were starved up to a period of 48 hours. Observations were made during this period on the healing of rickets, changes in citrogenase activity and citrate and total phosphorus in the epiphyseal cartilage.

2. Starvation induced healing of the rachitic lesion; maximum healing was observed at 48 hours.

3. The healing process was unaccompanied with increases in citrate or citrogenase activity in the epiphyseal cartilage. The total P content of the latter, however, increased significantly up to 36 hours.

4. Starvation of rachitic rats caused a fourfold increase in the plasma citrate.

5. The significance of the above findings has been discussed. A tentative hypothesis of a common pathway of action of starvation, citrate and vitamin D in the healing of rickets has been presented.

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A FOLLOW-UP STUDY OF FIFTEEN CASES OF 'KWASHIORKOR'.

BY

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THOUGH a considerable volume of literature on various aspects of the syndrome of protein malnutrition, known as 'Kwashiorkor', has accumulated, reports of detailed and long term follow-up studies of cases of this disease appear to be scarce. Altmann (1950) reported the results of a follow-up of 18 cases of the disease, but the duration of the follow-up did not exceed two years in any case. Ramanathan *et al.* (1955) followed up one hundred cases of kwashiorkor for periods ranging from 6 months to 5 years after discharge from the hospital; the study was mainly intended to find out the true incidence of relapses in the disease and did not include investigation of the histopathological features of the liver. Achar (1956) carried out a follow-up investigation, including liver biopsy studies, of 17 cases for 4 to 8 years after discharge from the hospital. Suckling and Campbell (1957) have reported a study of a five-year follow-up of 27 children with kwashiorkor in Cape Town.

Complete investigation, including liver biopsy studies of cases of kwashiorkor, followed up for several years, might be expected to throw light on the significance of the liver changes which constitute a striking feature of the disease in its acute stages. The occurrence of profound changes in the liver in cases of kwashiorkor, and the relatively high incidence of cirrhosis of the liver in regions where protein malnutrition is widespread, have to lead to the speculation that kwashiorkor in childhood might lead on to cirrhosis of the liver in later years. The question of the relationship between kwashiorkor and cirrhosis is linked with the fundamental problem of the exact relationship between parenchymal damage and mesenchymal proliferation in the liver and the precise rôle of protein malnutrition in both these

phenomena. A careful long term follow-up study of cases of kwashiorkor might be expected to contribute to the elucidation of these problems.

MATERIAL AND METHODS.

Fifteen children between the ages of 2 and 10 years formed the subjects of this investigation. All the cases had suffered from kwashiorkor at periods ranging from 9 months to 8 years before the present investigation. During the period when the cases were actually suffering from kwashiorkor, they had been admitted to the hospital and investigated by us. They were treated for 4 to 6 weeks in the hospital with a diet supplying 60 g. protein in the form of skim milk and 1,400 to 1,500 calories daily, and discharged when the oedema and the acute symptoms had subsided. At the time of the discharge, the mothers of the children were advised about the proper feeding of the children. It may be mentioned here, however, that for various reasons, the mothers were not able to follow this advice for long, and sooner or later the children reverted to their usual dietaries. Thirteen of the 15 children had been investigated in the earlier follow-up study also (Ramanathan *et al.*, *loc. cit.*).

In the present study, a complete clinical examination of the children was carried out. Heights and weights were accurately recorded. Total serum protein concentration as well as serum albumin concentration were determined by the micro-Kjeldahl procedure. Twenty-eight per cent sodium sulphite was used to fractionate the albumin (Cohn and Wolfson, 1948). Serum cholesterol levels, total as well as the ester fraction, were determined by Bloor's method. A complete hæmatological examination, which included the red cell count, packed cell volume and hæmoglobin determinations, was carried out.

Liver biopsies were performed through the transthoracic route using the Vim Silvermann needle with the usual precautions. The biopsies were done 1 to 2 hours after breakfast. The liver specimens obtained were fixed in 10 per cent formal saline and processed through paraffin. Sections were stained by hæmatoxylin and eosin and for reticulum by silver impregnation. Whenever the specimen was long enough, a portion was fixed in absolute alcohol and processed through carabowax for the histochemical demonstration of glycogen and alkaline phosphatase as described by Sriramachari and Ramalingaswami (1952).

Two cases which showed changes characterized by a moderate degree of protein deficiency type of vacuolation were administered a daily supplement of 30 g. protein in the form of skim milk for a period of 45 days and the investigations including liver biopsies were repeated.

RESULTS.

The results of the investigation are set out in Table I.

On careful questioning, it was found that two of the fifteen children had had a relapse of kwashiorkor subsequent to the first attack and obtained treatment for the same elsewhere. The relapses had occurred within 6 months after the first attack in case No. 3, and within 15 months after the first attack in case No. 7.

TABLE

Clinical and biochemical findings in 15 'follow-up' cases of kwashiorkor.

Case number.	Height as per cent of normal*.	Weight as per cent of normal*.	Moon face.	Skin changes.	Hair changes.	LIVER:			SERUM PROTEINS:			SERUM CHOLESTEROL:		
						Enlargement.	Protein deficiency vacuolation.	Mesenchymal reaction.	Total.	Albumin.	Globulin.	Total.	Ester.	Frec.
1	97	104	+	—	—	—	±	+	6.7	4.0	2.7	—	—	—
2	100	97	++	++	++	—	++	—	9.1	4.2	4.9	210	160	50
3	94	76	+	++	++	+	++	—	7.7	3.9	3.8	—	—	—
4	87	81	—	++	—	+	+	+	7.7	2.6	5.1	232	163	69
5	97	100	—	—	—	—	+	—	7.5	3.2	4.3	220	150	70
6	96	100	—	—	—	—	+	—	7.3	4.0	3.3	—	—	—
7	92	79	+	+	—	—	—	—	7.3	3.3	4.0	90	50	40
8	95	82	++	++	+	—	+	—	7.3	3.9	3.4	90	60	30
9	95	81	++	++	++	—	—	—	7.5	4.6	2.9	266	191	75
10	93	95	+	+	+	—	+	—	8.0	5.3	2.7	216	150	66
11	105	141	—	+	—	—	—	—	8.0	4.2	3.8	220	130	90
12	103	95	—	++	—	—	+	—	8.3	4.4	3.9	225	160	65
13	102	99	—	+	—	—	+	—	8.3	4.4	3.9	212	145	67
14	92	113	+	—	—	—	±	+	6.9	4.4	2.5	170	100	70
15	93	83	+	+	—	—	+	—	7.8	4.6	3.2	170	100	70

*The 'normals' referred to here are the community averages and are not truly normal.

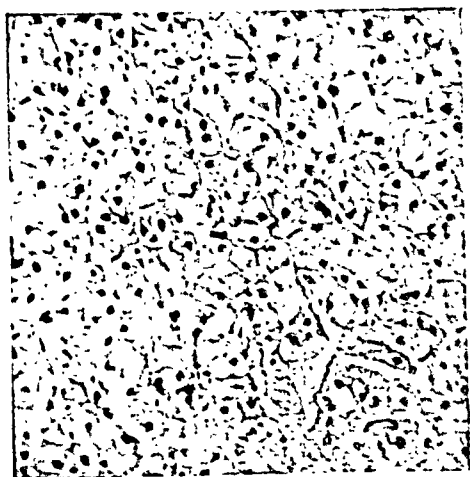
Clinical.—The height and weight records revealed that only 9 out of 15 children approached the averages observed for apparently healthy children of the same socio-economic group (Rao *et al.*, 1954). It must be pointed out, however, that even these so-called averages for the apparently healthy children are considerably below the accepted Western standards. Six children exhibited considerable sub-normal growth as judged by the local averages pertaining to the socio-economic group.

Abnormal prominence of the cheeks of the type designated as 'moon face' (Gopalan *et al.*, 1954; Venkatachalam *et al.*, 1954) was present in five of the 6 children exhibiting marked growth failure. Signs of vitamin deficiencies were surprisingly absent in all but one child, who showed Bitot spots. Varying degrees of follicular hyperkeratosis were, however, encountered in 6 of the 15 children investigated. The liver was enlarged in two children both of whom had exhibited such enlargement even during their first examination at the time when they were admitted with kwashiorkor. On palpation, the livers in these two cases appeared firm and smooth.

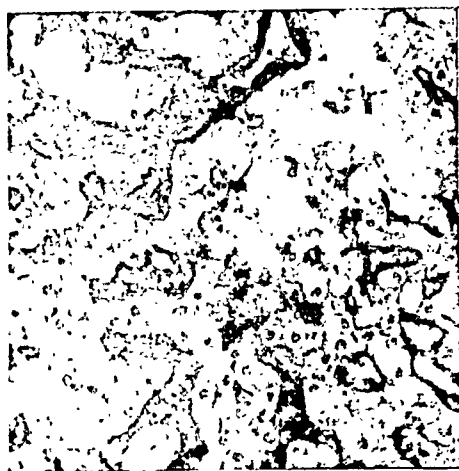
Biochemical.—Serum protein levels were generally in the normal range. Serum albumin concentration was somewhat reduced in 3 cases (Nos. 4, 5, 7), while the serum globulin concentration appeared slightly elevated in 4 cases (Nos. 2, 4, 5 and 7). Two children who showed low serum albumin levels also exhibited moon face and growth failure. Of the three children who showed a very mild degree of mesenchymal reaction in the liver, one (case No. 1) had normal serum chemistry. The second child (case No. 4) showed an abnormal

LEGENDS TO FIGURES.

- FIG. 1. Section of a liver biopsy showing severe degree of cytoplasmic vacuolation of the protein deficiency type. H & E $\times 100$.
- FIG. 2. Section of a liver biopsy with severe liver cell vacuolation showing an intense alkaline phosphatase reaction, particularly in the vicinity of the sinusoids. Gomori alkaline phosphatase $\times 100$.
- FIG. 3. Section of a liver biopsy showing cytoplasmic vacuolation of the protein deficiency type and a very tiny focus of cellular infiltration. H & E $\times 100$.
- FIG. 4. Section of a liver biopsy in the same case as in Figure 3 repeated after high protein therapy. The cytoplasmic vacuolation has disappeared. H & E $\times 100$.
- FIG. 5. Section of a liver biopsy from the same case as in Figure 1, showing a normal reticular framework. Gomori's reticulum $\times 100$.
- FIG. 6. Section of a liver biopsy showing a tiny cluster of mesenchymal cells around the portal tract. The parenchymal cells show varying degrees of cytoplasmic vacuolation. H & E $\times 100$.



1



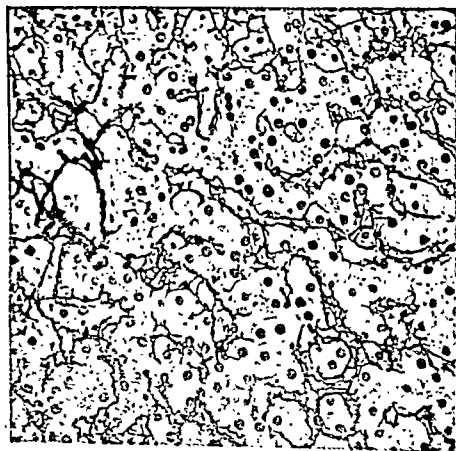
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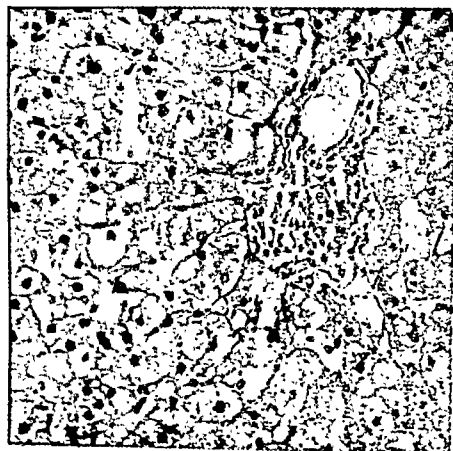
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serum protein pattern—the globulins were high and the albumin low. The ratio of free to total cholesterol, however, was normal. In the third child (case No. 14) while the serum protein partition was normal, the cholesterol esters formed only 59 per cent of the total as against the normal 68 to 70 per cent. It is interesting to note, however, that the protein intake in these three children (calculated as g. per kg. of body weight) is in no way different from the protein intake of the other children in these series.

Total serum cholesterol values ranged from 90 to 266 mg./100 c.c. (mean: 193.4) and the values for the ester fraction ranged from 50 to 163 mg./100 c.c. (mean: 129.8). These are in close agreement with the figures obtained by Ramanathan (1955) in 12 normal children of the same age and socio-economic group, but are considerably higher than those seen in children during the acute stages of kwashiorkor (Ramanathan, *loc. cit.*). The levels, however, appear to be generally higher than those reported by Behrendt (1949) for normal Western children of the same ages.

Diet surveys carried out by oral questionnaire on 11 of the cases indicated that the children were getting diets providing a daily caloric intake of 50 to 130/kg. and a daily protein intake of 1.3 to 2.4 g./kg. (mean: 1.85) almost completely derived from vegetable sources. On the basis of body-weight, the protein intake of the children might not be considered too inadequate. However, in view of the fact that there was evidence of considerable growth failure in most of the cases, an apparently adequate intake on the basis of body-weight could hardly be considered to meet the absolute protein requirements of these children. The calcium intake was of the order of 364 mg. and the iron intake of the order of 14.6 mg. daily.

Histological.—Histological examination of the liver biopsy specimens yielded the following results: Ten out of 14 sections contained one or more central veins and portal units. In the remaining five, the lobular relationships could not be ascertained as the specimens were rather small. In three specimens, the hepatic parenchyma was apparently normal. These included the livers of two children who were receiving nearly 30 g. of protein (derived from skim milk) as a supplement from the local church. The other twelve showed varying degrees of cytoplasmic vacuolation resembling the so-called plant cell-like appearance seen in malnourished livers (Gillman and Gillman, 1948; Srinivasaiah and Ananthachari, 1954). There was generalized swelling of liver cells associated with a reduction of the cytoplasmic mass and the nucleus was centrally situated (Plate IX, fig. 1). Fatty infiltration of the liver which is an outstanding feature of the acute phases of kwashiorkor (Srinivasaiah and Ramalingaswami, 1953) was practically absent in all the specimens. Sections stained by the periodic acid Schiff procedure, revealed very small quantities of glycogen. Thus, the vacuolation was neither attributable to fat nor to glycogen but probably represented a protein deficiency type of vacuolation of the liver cells (Ramalingaswami *et al.*, 1954). In some of the sections showing severe degrees of vacuolation, histochemical reaction for alkaline phosphatase was found to be increased (Plate IX, fig. 2). The intensity of reaction was most marked in relation to the sinusoids and is probably due to the mild degrees of intrahepatic obstruction caused by the swollen parenchymal cells. In the two cases where the liver biopsy was repeated after rehabilitation with high protein therapy the protein deficiency type of cytoplasmic vacuolation

completely disappeared (Plate IX, figs. 3 and 4). This gives added proof that the cytoplasmic vacuolation described in the present series of biopsies is a manifestation of a mild degree of chronic protein deficiency.

Mesenchymal changes were conspicuously absent in 12 specimens, and the reticular framework of the liver was practically normal (Plate IX, fig. 5). In three specimens, however, a very mild degree of mesenchymal change was noticeable. In these cases, small clusters of mesenchymal cells were seen occasionally around the portal tracts and also within the lobules (Plate IX, fig. 6). However, in neither of these cases was there any evidence of increase of extracellular connective tissue fibres. The two cases which showed this very mild mesenchymal reaction also were those that exhibited minimal parenchymal change.

There was no correlation between the presence of mesenchymal changes and the clinical enlargement of the liver.

DISCUSSION.

At the time of the follow-up, the dietary pattern of the children investigated was nearly the same as at the time of their first admission with symptoms of kwashiorkor. Evidently, under the compulsion of economic factors, it was impossible for the mothers to effect the dietary changes recommended to them. It was interesting that despite this lack of improvement of the diet only 2 of the 15 children have suffered from relapses. This lends further support to the view that in the actual development of the syndrome of kwashiorkor, dietary inadequacy of protein may not be the sole determinant (Gopalan and Ramalingaswami, 1955). The superimposition of acute infective episodes is probably necessary to tip the children into the florid state of kwashiorkor; in the absence of such episodes the children might be able to maintain themselves in a state of mild or marginal malnutrition as evidenced by the growth failure, the moon-face and the hypoalbuminæmia in several cases. Indeed the growth failure observed in these cases may actually be a means of adaptation of the organism to the protein inadequacy. A suggestive evidence in favour of this view was that while the body-weights of some of the children investigated here were definitely lower than even the averages obtaining for the apparently normal children of the same socio-economic group, and while the absolute daily dietary intake of protein was considerably less than the accepted normal allowances of children of comparable age, the protein intake considered in relation to the body-weight approached the recommended figure. The freedom from fresh attacks of frank kwashiorkor in a majority of the cases investigated, in spite of continuing dietary protein inadequacy, may be attributable to a relative diminution in protein requirement and relatively greater immunity to infections in the latter years of childhood. A survey of the morbidity of infants and children actually indicates that the period between 1 and 3 years is the period of maximal incidence of respiratory and alimentary infections in childhood; beyond the third year there is an appreciable decline in the incidence of these infections.

[An important finding in the present investigation was that none of the cases revealed evidence of hepatic fibrosis. / Although structural and functional damage of the liver in acute phases of kwashiorkor has long been recognized, it is not

known whether this leads to progressive or permanent liver changes. The present study in which cases had been followed up to periods extending to 8 years after the initial attack of kwashiorkor, revealed that fatty infiltration of the liver, a central feature of kwashiorkor, was absent in all cases. Even so, 12 of the 15 livers examined were not entirely normal but showed evidence of parenchymal damage attributable to protein deficiency. It is probable that this change is not a sequel to the attack of kwashiorkor but is independent of it. The striking absence of fibrosis in all cases indicates that in spite of the profound morphological changes in the liver at the height of the disease, kwashiorkor is not followed by permanent or progressive hepatic fibrosis eventuating in cirrhosis. These findings are in close agreement with those of Achar (*loc. cit.*), Suckling and Campbell (*loc. cit.*) and supported by the autopsy and biopsy studies of Higginson *et al.* (1957), who have concluded that fibrosis and cirrhosis rarely follows the fatty liver found in kwashiorkor. Brock (1954) has also commented, that the possibility of adult cirrhosis arising from kwashiorkor as a direct and necessary sequel must be regarded as unlikely. Altmann (*loc. cit.*), however, found evidence suggestive of imperfect recovery of the liver in nearly all the 18 cases he followed up. Six of them, in addition, showed abnormal cellular reaction in the portal tract. But evidence of frank cirrhosis was absent. Waters and Waterlow (1954) had also concluded that low grade nutritional liver injury of the type encountered here is by itself not sufficient to cause widespread fibrosis of the organ. This investigation supports the view that there is no direct relationship between the incidence of kwashiorkor and adult cirrhosis in a community.

SUMMARY.

1. The results of the follow-up study of 15 cases of kwashiorkor are presented. The investigation included a histopathological examination of the liver biopsy specimens of all these children.

2. Protein-deficiency type of vacuolation of the liver cells, which could be corrected by dietary protein supplementation was the predominant finding. Evidence of increased fibrous tissue formation was significantly absent.

3. Liver function, as judged by the serum albumin and serum cholesterol levels, appeared to be unimpaired.

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A PEDALLING ERGOMETER WITH A DYNAMO BRAKE*.

BY

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INTRODUCTION.

THE Pedalling Ergometer is a convenient apparatus for studying the physiological responses of human beings during exercise. If the design is satisfactory, it has many advantages as compared to other devices commonly used for work function tests, such as the tread-mill, stepping stool, etc. For example, this type of ergometer has minimum energy losses, and its grades of exercise are fixed, reproducible, and chosen with ease. In addition, prolonged exercise at moderate grades can be performed by a subject at constant rates without fatigue, and the caloric consumption of the person remains proportional to the exercise within a wide range. Physiological measurements are also facilitated because of the immobility of the upper portion of the body.

The early pedalling ergometers utilized a friction belt strapped around the rotor wheel as the brake, the mechanical torque being indicated by a pair of spring balances. The inherent disadvantages of this type of ergometer are: (a) Inaccuracy and lack of sensitivity of the spring balances available, (b) the difficulty of setting the desired grade of exercise, and (c) the slackening of the belt due to wear. However, a recent design by von Döbeln (1954), using the sinus balance technique, facilitates setting and measurement to a great extent.

*Condensed from Industrial Health in India Series No. 70.

In the Krogh (Krogh, 1913, described by Rao, 1945) ergometer in which the principle of eddy currents is used for braking, electrical energy is used for varying the grade, and the torque is measured by means of counter-balancing lever and weights. Although this design is one of the first with which accurate measurements of the torque could be made, it has the drawbacks that the procedure for setting a grade of exercise is elaborate, and the criterion of satisfactory performance is subjective. In order to avoid these difficulties, the work may be converted into an easily measurable form of energy, as, for example, electrical energy. This electrical energy output may itself be measured to specify the grade of exercise. Ergometers based on this principle have been made by Kelso and Hellebrandt (1934), Meneely and Kaltreider (1941) and Tuttle and Wendler (1945). Müller (1952) has described the conditions governing good pedalling ergometers. He has also presented the details of construction of one in which a flat copper ring rotating between the poles of a strong permanent magnet provides the brake.

In this Laboratory a pedalling ergometer suitable for low grades of exercise which could be easily preset, maintained constant, and reliably reproduced, has been constructed.

PRINCIPLE.

The conversion of the work performed by the subject into electrical energy is achieved by coupling a dynamo to the crank pedal. The efficiency of such a conversion is usually more than 90 per cent. When a load is connected to the generator the power dissipated is a measure of the work performed by the human subject, or, in other words, the physiological stress varies directly as the power dissipated in the external circuit of the generator. The output of a generator depends upon the speed of rotation of the armature, the magnetic flux of the field and the electrical resistance of the external circuit. Variation of any one of these leads to different exercise grades.

Generally, continuous work at a rate of 90,000 ft. lb./hr. is considered to be strenuous for a human being (Brandt, 1947). In terms of electrical units this is equal to 33.9 watts. Therefore, the total capacity of the generator used need only be of this order. It is, however, advisable to use a generator of comparatively higher capacity in order to avoid probable heat effects.

CONSTRUCTION AND CALIBRATION.

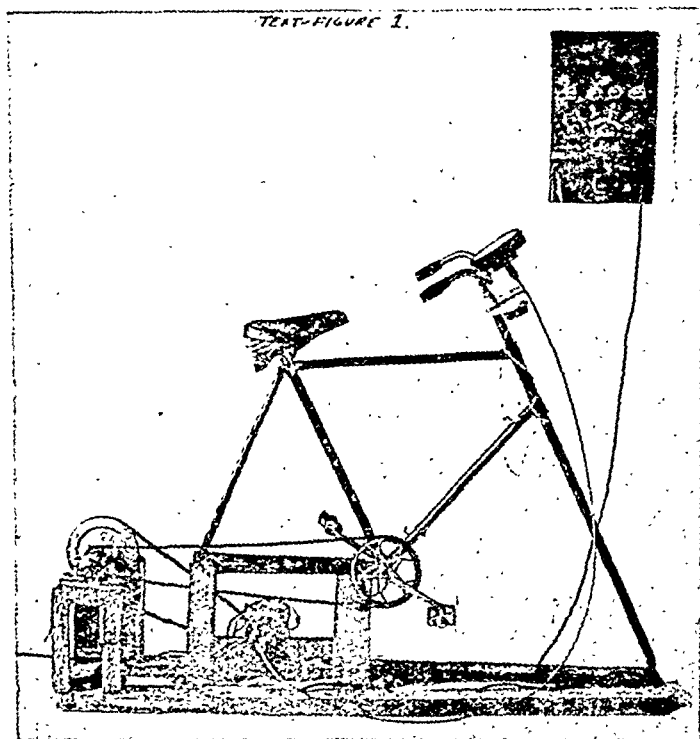
Text-figure 1 of the ergometer illustrates the details of construction. Special attention has been given in the design towards the reduction of friction and inertia by the use of ball bearings for the wheel shaft, a light aluminium pulley for coupling the generator, a flywheel on the generator shaft for facilitating smooth revolution and by proper spacing and mounting of the components for stability and non-vibration. The speed of pedalling is readable on a revolution indicator attached to the dynamo shaft.

The generator used in this ergometer is the CAV automobile dynamo [type D45DN (NV)] whose field coil is excited by the D.C. mains through a series resistance. The output voltage of the dynamo is 12 volts and this may be

dissipated either by 12V automobile lamps of different wattages or by wire-wound resistances, whose values are chosen such that the power consumption increases in uniform steps. The electrical connections are shown in Text-figure 2:—

TEXT-FIGURE 1.

A pedalling ergometer with a dynamo brake.

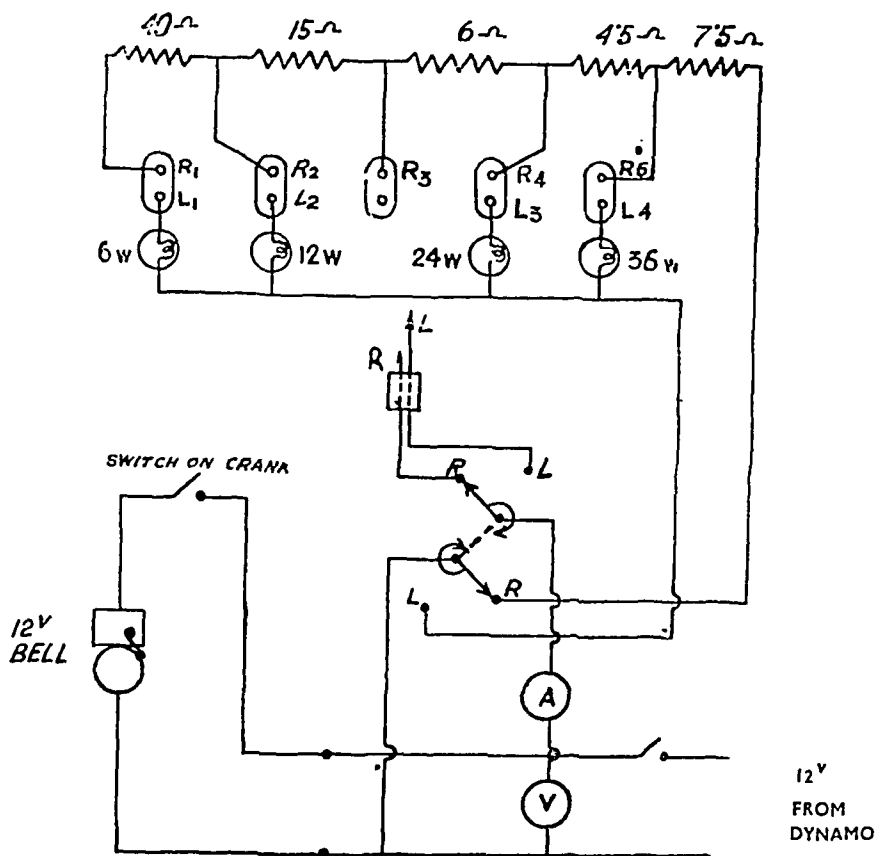


To mark time and to help synchronize the pedalling rate with a metronome, a 12 V electric bell is included in the output circuit. A spring strip on the pedal crank acts as a switch for the bell, which makes a tinkle at every push of the pedal.

In this type of ergometer, the output wattage, or in other words, the work-stress, for any suitable external load can be varied either by changing the pedalling frequency or by altering the field magnetization current. Using both these methods, the performance of the ergometer has been extensively tested and calibrated. The study of its output characteristics under different conditions and with different human volunteers pedalling it, yielded the following results:—

(1). The lamp resistances do not give uniform wattage steps, despite their being uniform by rating. The wire-wound resistances, however, have linear load—output relationships for all pedalling frequencies.

TEXT-FIGURE 2.

Circuit diagram of the lamp and resistance loads used with the ergometer.

(2). Low field magnetization currents give a more pronounced linearity; but there is a lower limit since too low currents fail to give adequate workload stresses in the desired range.

(3). There is an optimum pedalling frequency at which the wattage is constant for different voltage outputs. Working near this optimum would result in negligible variations of the wattage output despite small fluctuations in the constancy of pedalling rates.

(4). It is possible to obtain higher outputs than the maximum designed for by enhancing the pedalling frequency in suitable steps. For example, the Graph shows how the output wattage, with a resistance load in circuit, rises along a parabolic path when the pedalling frequency is increased.

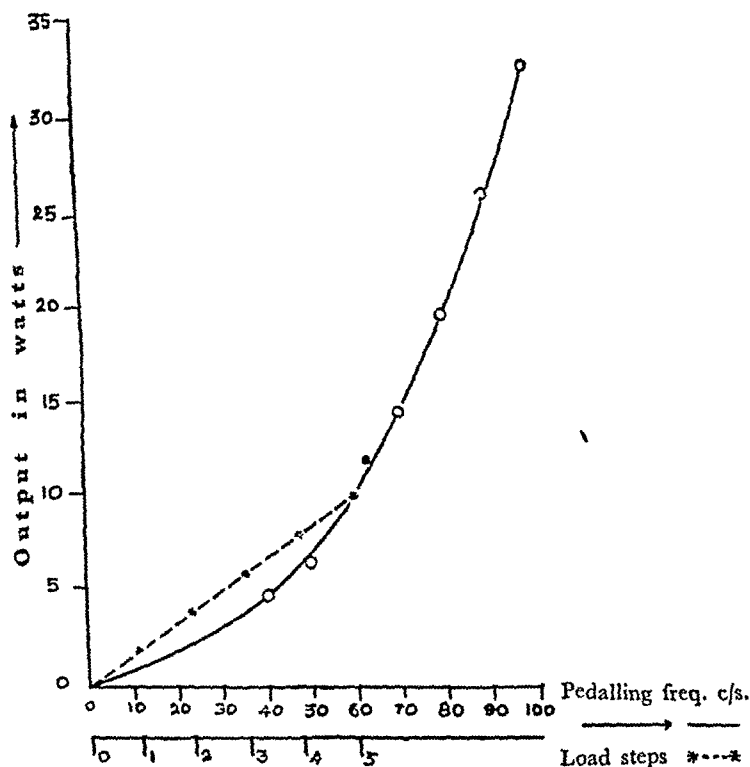
(5). Repeated test-runs with different volunteers yielded consistent results, thus giving an assurance of the reproducibility and reliability of the present design.

This ergometer provides five resistance load steps dissipating 2, 4, 6, 8 and 10 watts, with an optimum pedalling frequency of 60 rpm and a field current of 1.3

amps. (Graph, dashed line). Outputs beyond 10 watts, up to a maximum of 32 watts, are achievable by increasing the pedalling frequency.

GRAPH 1.

Output in watts vs. pedalling frequency for a fixed resistance load.



DISCUSSION.

The working of the ergometer is quite simple, the main criterion of its satisfactory performance being the pedalling frequency. This is subject to the control of the human operator who is required to synchronize the electric bell on the ergometer with the metronome. The revolution indicator mounted on the handle-bar is a further guide to uniform pedalling. It is found that optimum operating speeds can be easily maintained by the volunteers for prolonged periods of time at the loads used. Even an untrained subject can quickly achieve and maintain a constant rate of pedalling with the help of the simple accessories provided. One of the tests has shown that small departures from constancy of the optimum pedalling frequency do not affect the output voltage significantly.

A notable feature is that the changing of load steps is made easily, without disturbing the volunteer, by changing the jack-pin to the proper socket. The tests of calibration performed in the series of experiments clearly indicate the ease of reproducibility of the load steps. The output is independent of the volunteer and remains constant as long as the condition of optimum pedalling frequency is maintained. Therefore, continuous wattage measurement is unnecessary. However, plug-in sockets for a voltmeter and an ammeter are provided to enable periodic checking of the calibration.

This electrical pedalling ergometer is superior to the conventional friction type of ergometer in the matter of setting the loads and reproducibility. Moreover, it compares favourably with other designs using the principle of dynamo braking, such as that of Kelso *et al.* (*loc. cit.*). The essential departure here from the design referred to above is the use of separate resistance steps for the different loads. This renders the setting easy and independent of the human element. Obtaining different load steps by changing the pedalling frequency and/or the field magnetization current as in their case is not a simple procedure. For, the handling of the large field currents involved and the maintenance of odd values of pedalling frequency make this technique unsafe and difficult. Besides, voltmeters and/or ammeters, a matter of necessity in that design become superfluous in this case. The calibration is also simple in comparison and need not be repeated often.

Müller (*loc. cit.*) and Lehmann (1957) have pointed out that the heat losses in the dynamo may introduce a factor of uncertainty in the output due to the temperature coefficient of resistance of the copper windings. The use, however, of a comparatively large capacity generator, as in the present instance, for yielding a low output precludes this difficulty almost totally. And this is evident from the observation that the output wattage is maintained constant over prolonged periods of performance.

A preliminary study of the metabolic cost for the performance of different grades of exercise on this ergometer was made for four volunteers by the oxygen-consumption method. On the average, the oxygen consumption held a linear relation with the work grade, thereby indicating satisfactory correlation between the physiological work stress and the electrical wattage output.

A difficulty common to all pedalling ergometers is the unaccounted mechanical losses in the transmission system. In addition, ergometers using dynamo braking have some electrical losses due to normal armature reaction, etc., in the dynamo. As the total losses in this machine have not been determined, no conclusion regarding the absolute work output of the volunteer can be made at this stage. The measured wattages, however, are almost fully a measure of the total work done. For, literature shows that the total losses in a carefully designed pedalling ergometer of this type are of the permissible and accepted order of 5 per cent. It is, therefore, apparent that the application of this ergometer would be most satisfactory where relative physiological studies are to be made.

This ergometer is chiefly designed to provide low grades of physical exercise for stress physiology experiments. Human volunteers can perform at these levels for long periods without getting excessively fatigued. In the higher grades of exercise, however, there is a limitation placed by the incapacity of the human volunteer to maintain constantly high pedalling frequencies for long periods.

It may be noted that materials and equipment that are used here are readily available and inexpensive; the total cost being only about Rs. 500/-.

SUMMARY.

1. A pedalling ergometer is designed and constructed to meet the need for a machine suitable for prolonged exercise against low grades of work with a high degree of reliability, reproducibility and ease of operation.

2. For adequate calibration, various tests are designed and carried out. The ergometer, as designed and constructed can be used to provide work load stresses corresponding roughly to 4, 10, 13, 14, and 15 watts in the case of lamp loads, and 2, 4, 6, 8 and 10 watts in the case of resistance loads with a pedalling frequency of 60 rpm. The resistance loads give more uniform steps than the lamp loads. They can also be used to give higher work loads than mentioned above up to a maximum of 32 watts. Preliminary studies of the oxygen consumption of different volunteers at the different loads indicate the proportionality between work load and metabolic cost.

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A HEALTH-PHYSICAL SURVEY OF MEDICAL X-RAY INSTALLATIONS IN CALCUTTA*†.

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INTRODUCTION.

SWEEPING comments and categorical opinions are often expressed regarding the indifferent attitude of radiologists to x -ray exposure. Facts, however, are very difficult to find. The dangers of such exposures, though nothing new, have, of late, assumed a new dimension thanks to the enhanced applications of x -rays for medical purposes and to the development of new and powerful radiation sources. The recent advances in the field of nuclear energy production and application have focused the lime light on the dangers of the use of ionizing radiations for a variety of purposes. The investigations prompted by many scientific bodies, such as the British Medical Research Council and the United States National Academy of Sciences, etc., have gone a long way towards the clarification of the ultimate hazards. An interesting fact that emerged from these studies was that the medical use of x -rays contributed to public danger no less than 22 times as compared to the much wonted atomic energy operations. This finding amply justifies the many recommendations and regulations that have been put forward by the Radiological Commission towards safe procedure in the use of x -rays for medical purposes. So much so, it has become customary for almost all progressive countries to have some organization for the protection of personnel using or handling x -rays or radio-active materials.

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In India, an entirely different picture is discernable. The use of x -rays for medical diagnosis in India dates back to the year 1908 when the first unit was started in the Medical College, Calcutta. Since then, x -ray practice has become an indispensable part of medicine. A recent estimate puts the number of x -ray sets used for medical purposes only in India between 3 and 4 thousand. Reliable reports of radiation injuries amongst x -ray personnel in India are not, however, available in literature. It is, however, common knowledge that most radiologists develop some abnormality of the skin, especially of their hands towards the end of their career. Haemopoietic injuries are also said to have occurred although details of such cases could not be obtained. A recent case of fatality, alleged to have been caused by radiation exposure over a life-time, provokes serious concern. Equally unavailable are data regarding the magnitudes of exposure to x -rays of either the operating personnel or the public.

The past history of radiation injuries in India, obscure though it be, and the present trend of increasing use of radio-diagnosis and therapy pose before us the serious problem of radiation hazards. India does not have an organization responsible for the control of the use of x -rays for medical purposes. Health-physics facilities are also totally lacking except in a few isolated cases. Experience in other countries has shown that radiation can be handled safely without any risk whatsoever. Avoidance of radiological hazards in the medical facility is absolutely essential. This study is an attempt to set the ball rolling by assessing the gravity and extent of radiological hazards in x -ray installations in Calcutta and evaluating the current status of the problem.

Experiences in recent years in the field of radiological protection have revealed the usefulness of adopting the occupational health or industrial hygiene point of view in the study of radiation hazards. Radiation hazard problems bear a high degree of similarity to the conventional health problems in industry. This is specially remarkable in the matter of evaluation, critical limits and control measures. Hence, the present study is also coloured by the occupational health point of view so as to yield better results.

EXPERIMENTAL PROCEDURES AND RESULTS.

In this pilot study 15 medical x -ray installations in Calcutta were surveyed for possible stray radiation. These included:

- (a) One roentgen therapy unit,
- (b) Four radiographic units,
- (c) Two fluoroscopic units,
- (d) One dental radiographic unit, and
- (e) Seven units for both fluoroscopy and radiography.

The operating characteristics of these units ranged from 70 to 150 kV. and 15 to 500 mA. In each of these installations the environment was monitored for stray radiation by the ionization dosimetric method. Simultaneously, the status of the general environment and current x -ray room practice were estimated. Besides this environmental survey, the personnel of these installations were subjected to medical and haematological examinations in order to detect possible haematological damage incurred by them due to radiation exposure.

A. *Evaluation of the general environment.*—In evaluating the general environment, stress was given to the factors laid out by the International Commission on Radiological Protection. These factors are: ventilation, lighting, temperature and humidity, lay-out of the equipment and design of the x-ray room, electrical wiring and location of master switch. In no case, the general environment could be considered ideal for the practice of radiology. In most cases the main defect was lack of space, which prevented adequate or satisfactory lay-out of equipment as well as sitting arrangement for technical and non-technical personnel and patients. Installation of x-ray chambers in any room available, as seems to be the practice at present, must be curbed.

B. *Evaluation of the installation and protection practice.*—The status of the equipment was evaluated in a qualitative manner by noting the date of manufacture and the physical condition of the x-ray tube. Tube housing protection was assumed to be satisfactory inasmuch as most commercial x-ray tubes more than meet the International Commission's recommendation: 'A fully protective tube housing is one in which the direct radiation, i.e., the leakage radiation in the housing is reduced to 6.25 mr./hr. at contact with the tube housing when the tube is continuously operated at its maximum rated current for the maximum rated voltage'. Barring two x-ray installations, viz., (1) Installation I—the unit was bought from army disposal with the cone restricting the useful beam missing, and (2) Installation K—a portable unit of obsolete design, the installations are of fair condition. A fact that merits special mention is that there seems to be an almost complete lack of maintenance in most of the installations.

The protection practice in these installations was assessed by noting: (i) the type of shielding in the installation, (ii) availability and use of protection equipment, such as lead gloves, lead aprons, etc., (iii) distance of personnel during exposure, (iv) the presence or absence of scatterers, and (v) knowledge of radiation hazards amongst the personnel. It was observed that auxiliary protection devices, such as lead gloves and lead aprons or lead screens are not generally available and even if available, they are rarely resorted to by the technicians, even when they had to expose themselves to the direct radiation, as for example, when helping patients during radiography. In no case was the installation isolated from other working rooms nor did the walls have any protective covering. As regards the knowledge of hazards, the auxiliary staff and technicians had little or no realization of the radiation hazards. Paradoxically, however, the technicians fully comprehend the consequences of keeping unexposed films in the x-ray room. Distance from the x-ray source is primary in the avoidance of exposure. But though the control board where the operating technician is expected to be, are usually 1 to 2 meters from the x-ray tubes, it was rarely that the technician or other staff took care to be as far from the radiation source. Once again, the shortage of space resulted in the cluttering up of the x-ray rooms by disused equipment, furniture, etc.

Measurement of stray radiation.—In the present investigation importance was given to area monitoring rather than personnel monitoring. Apart from measuring the radiation dose-rate at a standard reference position estimation of the dose-rate was also made at the control board where the operating technician is expected to remain during the x-ray exposure. For this purpose, a quartz fibre individual dosimeter which may be worn in the pocket was used. The dosimeter

has a range of 200 mr. and measures the integral dose received over a period of time. These dosimeters are designed for working conditions where gamma rays and x-rays are encountered and have a natural leak of less than 2 per cent in a day and an accuracy of ± 10 per cent.

In the estimation of the environmental radiation the time of exposure is as important as the instantaneous radiation intensity; for the assessment of the hazards is to be made on the basis of the integrated dose-rate. Therefore, the charged dose-meter was kept at a standard reference position with respect to the x-ray tube till appreciable readings were obtained. During this period, the times of exposures given with the x-ray tube were also recorded. The standard position was 1 meter in the transverse direction from the x-ray tube in the horizontal plane. At this position the radiation dose per minute of x-ray exposure was computed from 3 or more different readings taken at various times. All readings were taken under actual conditions of work, i.e., when persons were being x-rayed. Since no significance was given to the influence of kV. or mA. on the tube output, these values do not give the absolute dose but rather the effective dose, i.e., the amount of radiation absorbed by the dosimeter. This may be taken to approximate to the first degree the tissue absorption at that point. These readings at the standard position enable the comparison of the radiation environments in the different installations. The results of these measurements are presented in Table I:—

TABLE I.

Radiation dose-rate at a distance of one metre in the transverse direction.

Installation.	mr./min.	Average exposure time min./week.	mr./week.	Number of working days/week.	mr./day.	mr./week.
A ₁	12.20	6.15	75.03	6	...	...‡
A ₂ *	26.90	9.60	258.40	6	...	...‡
B	83.40	4.26	355.28	6	55.0	330.0
C	151.30	1.00	151.30	6	25.0	150.0
D	81.50	1.50	122.50	6	25.0	150.0
E	106.30	2.00	212.60	6	39.0	234.0
F	744.70	3.00	2,234.10	Not in regular use		
G ₁	97.40	5.25	511.35	6	89.0	534.0
G ₂	38.40	3.21	1,451.98	6	253.0	1,518.0
G ₃ †	1.39	157.80	112.05	6	22.0	132.0
H	133.30	0.50				
H ₁ †	22.70	2.00				
I	72.90	1.20	87.48	6	16.1	96.6
J†	4.40	210.00	924.00	6	141.2	847.2
K	19.67	2.00	39.34	6	7.0	42.0
L	2.22	7.00	15.54	2	7.5	15.0
M	4.78	1.00	4.78	6	...	...‡

*Therapeutic; †Fluoroscopy; ‡Aggregate daily readings not taken.

Radiation is present in the environment only during the operation of the x-ray tube. Therefore, these values were calculated on the basis of mr./min. of

α -ray exposure. Computation of the weekly dose rate has been made from the average exposure time in minutes per week in each of these installations and shown in this table. However, it may be convenient to take the integrated dose per day for calculating the weekly exposure. This has also been done and the values obtained are given in the same table. The fair agreement of the two weekly values demonstrates that it is not absolutely essential to determine the actual exposure time of the α -ray tube and compute the radiation dose-rate. However, the knowledge of the actual exposure time enables the limitation of the total weekly exposure time so that the weekly radiation exposure does not exceed the recommended limit. It will be seen from Table I that the radiation dose-rate in the different installations vary widely. In the fluoroscopic units (G_3 , J, L, H_2) the radiation dose per week is small; this is because the tube current which controls the number of electrons bombarding the target per second and, therefore, the quantity of α -rays produced is small, being only of the order of 5 to 25 mA. In many installations (B, F, G_1 , G_2), the radiation dose is excessively high. The highest radiation intensity was observed in installation F; this may be because the photo-fluorographic technique used here necessitates an intense α -ray beam (i.e. high milliamperage) in order to obtain clear miniature skiagrams.

Using the inverse square law of radiation attenuation, the radiation dose at the control board was calculated from the standard position value. If this value was large enough to be measured within a short time, the dosimeter was kept either in the operator's pocket or on the control board during the actual course of work and the mr./min. value for this position was also obtained as before. In the case of installations where the amount of work is very small or where the 1 meter reading itself was low such measurements were not attempted. The results of these measurements are shown in Table II. The calculated and observed values are seen to agree as far as can be expected from this technique. The reason for the lower observed value in the case of fluoroscopy is that the operator is shielded by the lead glass of the fluoroscopic screen. In general, this method of computation of the dose-rate of the operator is satisfactory despite the limitation of the inverse square law for continuous or white radiation and the limitation due to radial distribution, both of which were neglected in the computation. This is especially true in such installations where:

- (1) The stray radiation at 1 meter is small.
- (2) The kV. range used is below 100 kV.
- (3) The time of operation of the tube is small.
- (4) The control board is in the near-transverse direction.

The average weekly exposure time was computed in these installations on the basis of observation for a few days. In installation F, however, the time of 3 minutes in the table is the maximum possible because the work in this unit is intermittent and computation of weekly time could not be made. The exposure received by the operator is the product of the radiation dose-rate and time of operation of α -ray tube. This is the value on which the degree of hazards depends and, therefore, should not exceed the maximum permissible limit. The exposure received by the operator per week both on the basis of the mr./min. reading as well as the mr./day reading are given in Table II. Here again, the agreement between

the two sets of values justifies the use of the day as the unit of time for the assessment of hazards rather than the tube exposure time. Whereas in most of the installations, the weekly exposure values are definitely low, installations F, G₂ and B show significantly high radiation dose-rates. For the benefit of the participating installations, further measurements were made at places, where the radiation dose-rate may be very high. Table III gives these values as well as the gist of the appropriate recommendations made to these installations.

TABLE II.

Radiation dose-rate at the control board.

Installation.	mr./WEEK.		Average exposure time/week min.	Exposure received by operators Mr./week.	Working days/week.	mr./day.	mr./week.
	Cal.	Obs.					
A ₁	1.81	...	6.15	11.1 [†]	6	...	...
A ₂ *	2.20	1.60	9.60	15.36	6	...	...
B	18.91	64.79	4.26	276.00	6	60.00	360.00
C	67.24	...	1.00	67.24 [‡]	6	11.10	66.60 [‡]
D	20.38	...	1.50	30.57 [‡]	6	5.17	31.02 [‡]
E	26.80	...	2.00	53.60	6	9.75	58.50 [‡]
F	206.30	220.00	3.00	660.00	...	...	...
G ₁	22.10	22.50	5.25	118.00	6	32.68	196.08 [‡]
G ₂	83.10	69.90	3.21	358.50	6	39.00	294.00
G ₃ *	1.64	0.85	17.80	122.80	6	(10) [‡]	105.00
H	110.08	80.00	0.50				
H ₁	40.49	41.40	2.00	132.30	6	...	...
I	50.62	57.00	1.20				
J	1.74	0.63	210.00	21.30	6	2.20	13.20
K	8.74	10.65	2.00	4.34	2	5.00	10.00
L	3.95	0.62	7.00	9.75 [‡]	...	...	...
M	9.75	...	1.00				

*Therapeutic;

†Fluoroscopy;

‡Calculated values.

It is our experience that this technique of measurement at a standard distance first, followed by measurement at different places, if necessary, is very effective for obtaining the maximum information within a short time. It must also be emphasized that each measurement was completed in a single period never exceeding 1 hour, so that the leakage and background effects are negligible and that all readings were taken during the normal radiological work in the installation.

When the survey of any installation was completed, the results of the observation were communicated to the Radiologist-in-Charge. In the reports, the degree of hazard, areas of high dose-rates, suitable precautionary measures and suggested recommendations for the protection of the personnel were incorporated. In making these recommendations a maximum permissible limit of 500 mr./week was adopted without prejudice to the currently accepted MPL of 300 mr./week inside the dorsal layer of the skin for radiation exposure. It was considered advisable to relax the MPL for the following reasons:

- (1) The measurements were made in air.

- (2) The maximum working period of the persons in these installations is 4 hours per day for 6 days a week as compared to the 8 hours 5-day week taken as standard for recommending the above limit. Hence, there is a larger recovery time between exposures.
- (3) Operating voltage of the x-ray tube is usually less than 100 kV. and, therefore, the penetrating power of the x-rays is low.

TABLE III.
Areas of high radiation dose-rates.

Installation.	Description of area.	Distance from x-ray source, metres.	Intensity mr./week.	Recommendations.
C	The region between the x-ray tube and chest stand.	...	Primary useful beam	As this region is flanked by doors leading to radiologists' room and to the verandah, the use of this area as a passage should be restricted. It is preferable that the chest stand is placed in another direction, e.g., the present position of control board. Protective barrier required here.
D	Door separating the nurses' quarters (West wall).	3.0	282.5	
F(i)	A region in the direction of the entrance door.	1.0	1,896.0	Remaining inside x-ray compartment during radiography is unsafe.
F(ii)	Door leading to driver's compartment	3.0	540.0	The driver should not occupy his seat during radiography.
G ₁	In the direction of entrance exit door (patients crowd around here).	1.5	1,605.0	The patients should not be allowed inside the x-ray room.
G ₂	Region near entrance door. (during chest radiography).	6.2	1,312.0	A lead barrier to cut off the primary beam is to be placed behind the chest stand or the orientation of the beam changed.
H(i)	Waiting room	1.8	1,850.0	High intensity radiation is present in the forward direction of x-ray tube.
H(ii)	Partition between the wait-room and x-ray room.	1.9	875.0	Protective barrier is necessary.
I	The region where technicians stand for holding the patients during radiography.	1.0	6,841.0	Lead barriers to shield person helping patient essential.
J	A circular region of radius 1 m. around the x-ray tube.	1.0	924.0	Nobody should stay within this continuously during exposures.

Medical and clinical examinations.—Apart from the general examination as to the status of health and recording the occupational history, particular attention was given to the examination of the skin and its appendages and to a detailed blood examination for any marked abnormality attributable to radiation exposure. A total of 60 personnel working in the various x-ray installations were examined. Their periods of service in x-ray work ranged from 20 years to 4 months, the average being 6 months. Of these, 32 were medical men, 15 x-ray technicians, 10 attendants and orderlies and 3 nurses. The only case of visible radiation injury

the two sets of values justifies the use of the day as the unit of time for the assessment of hazards rather than the tube exposure time. Whereas in most of the installations, the weekly exposure values are definitely low, installations F, G₂ and B show significantly high radiation dose-rates. For the benefit of the participating installations, further measurements were made at places, where the radiation dose-rate may be very high. Table III gives these values as well as the gist of the appropriate recommendations made to these installations.

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H ₁	40.49	41.40	2.00	68.40	6	10.00	60.00†
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J	1.74	0.63	210.00	132.30	6	...	...
K	8.74	10.65	2.00	21.30	6	2.20	13.20
L	3.95	0.62	7.00	4.34	2	5.00	10.00
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It is our experience that this technique of measurement at a standard distance first, followed by measurement at different places, if necessary, is very effective for obtaining the maximum information within a short time. It must also be emphasized that each measurement was completed in a single period never exceeding 1 hour, so that the leakage and background effects are negligible and that all readings were taken during the normal radiological work in the installation.

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- (1) The measurements were made in air.

5. Periodical or, better, continuous checks of radiation exposure of the personnel are indispensable in protection practice. A standardized film badge technique or the pocket dosimeter method are suggested for this purpose. Records of the weekly exposure of all personnel throughout their working life should be maintained. In addition half-yearly haematological examinations for the staff and yearly area monitoring for radiation dose-rate are recommended. Whenever new machines are installed or the existing lay-out changed radiation surveys should be repeated.

6. Areas where a positive hazard exists should be indicated by red paint on the floor; this demarcating line will help remind the personnel to keep off this area during the radiological work. Moreover, safety regulations are to be prominently displayed within the x -ray room and the personnel should be persuaded to read the same at least once a month.

7. Annual vacation at the seaside or in the country for all radiological personnel is a beneficial practice.

DISCUSSION.

The picture painted by the current investigation must be considered to be somewhat reassuring in the light of the recommended maximum permissible unit. The primary reason for this fortunate situation is the fact that the present aggregate volume of radiological work in these x -ray establishments is still very small. The weekly hours of radiological work even in the major hospitals here are only 24 hours, as compared to the 40-hour week in hospitals in U.S.A., etc. A further illustration of the same point is that the average radiological work done in a year by all these hospitals and clinics is only approximately one-half of that done by a major hospital in the United States. There is no doubt that this circumstance will not prevail very long. For already the augmentation of radiological work in this country is highly significant. The application of x -rays for medical diagnosis and therapy is steadily increasing. The existing x -ray departments in all hospitals are rapidly expanding and new and more powerful x -ray machines are being installed. When taken in conjunction with the National and International Atomic Energy Operations and the potential application of nuclear energy for power production, the increased popularity and application of radiography with powerful machines provokes serious concern regarding the hazards to the personnel and perhaps with respect to possible effects on posterity. Recent research by the M.R.C. has pin-pointed the fact that 'uncontrolled and unlimited use of x -rays is to be strongly deprecated'.

Consequently, there is not much room for complacency inasmuch as there were work areas of high intensity which there is no guarantee were not used by the personnel. It is also to be pointed out that technicians and auxiliary staff do not understand adequately the potentialities of radiation as a health hazard. It is important that these personnel should be made to realize, accept and practice the fundamental philosophy of radiation protection, viz. 'avoid all unnecessary radiation exposure'.

Under the circumstances, the first thing that we may consider is the adoption of a uniform code for x -ray protection for the benefit of those occupationally exposed,

was observed in an orderly in installation D. Here, due to the absence of a cassette holder for taking chest x-rays, the orderly had to hold the cassette in position, thus exposing his hands to direct radiation. As a result the fingers of both the hands showed severe dermatitis, with desquamation and ulceration. This condition was noticed by the radiologist and the orderly was given 6 months leave. At the time of medical examination, the ulceration was healed and the only evidence was depigmented skin alternating with normal skin on the dorsum of the fingers. In three other cases, some roughness of the dorsum of the hand was noticed.

The examination of samples of blood from these personnel included the estimation of hæmoglobin and counts for the total r.b.c., total w.b.c. platelets and differential counts for leucocytes. Analysis of the blood pictures fail to show any marked abnormality on the whole. There were, however, 4 cases of rather pronounced eosinophilia, which could not be definitely attributed to radiation.

It is obvious that a single examination as has been done in the present study is of limited use for the estimation of radiological hazards, since the injurious effects of radiation are mostly cumulative and are apparent only after exposure over a continuously long period. Though practically the only useful biological examination available for early detection of radiation injury, blood examination lacks both specificity and sensitivity and, therefore, cannot be used with convenience for radiation monitoring. Moreover, the exposure record of these personnel could not be computed and as such the blood pictures could not be correlated with the amount of radiation received by them during their working life. However, pre-employment blood and clinical examination followed by the periodical blood examination of the personnel are bound to be useful in controlling the hazards, *provided they are performed in conjunction with health-physics monitoring of the environment.*

CONCLUSIONS AND RECOMMENDATIONS.

The major points of interest emerging from this study are outlined below:

1. A potential hazard due to exposure to radiation exists in most of the x-ray installations; and this is only augmented by lack of realization and recognition of the dangers by the technicians.
2. Faulty techniques, such as holding patients in position for radiography and attempting to use the available x-ray unit for work for which it is inadequate for etc. should be discouraged.
3. In the design of an installation careful thought must be given to the avoidance and minimization of radiation exposure by locating the x-ray unit in large, well ventilated and preferably isolated rooms. It is also emphasized that proper maintenance of the x-ray unit and the room are essential.
4. Proper shielding of the x-ray tube, adequate coning of the primary beam, provision of adequate protection equipment, such as lead aprons and lead gloves are indispensable in all installations. Further, if a large amount of work is customary, it is advisable to have a number of operators and attendants to follow a work shift system so that no person receives more than the permissible limit of exposure. All x-ray personnel should be trained to observe strict personal hygiene.

HÆMAGGLUTINATION BY STRAINS OF *ESCHERICHIA COLI* ISOLATED FROM CASES OF URINARY INFECTION.

BY

N. P. GUPTA,

CORRIGENDA

In paper entitled "Effect of Hypothermia on the Acetylcholine and Glutathione Content of Brain and Heart of Dogs" by B. K. Anand, C. L. Malhotra, Baldev Singh, P. G. Pundlik and G. S. Chhina, *Ind. Jour. Med. Res.*, 46, January, 1958, Table I on page 23, the decimal in all the figures should be shifted by one figure to the right.

March 1958.—Page 175, the heading of the Graph below figure 1, the word 'present' is superfluous.

Page 258, para 3, last line, the word 'problem' is superfluous.

Page 320, footnote to Table I, please read "*" and † Figures in table represent the average of three days measurements".

—Editor, *Ind. Jour. Med. Res.*

Agglutination of red cells

Agglutination of red cells by strains of *Bacterium coli* was reported as early as 1908 by Guyot. A number of strains of *Bacterium coli* was found by Rosenthal (1943) to possess agglutinating substance against red cells, leucocytes, thrombocytes, spermatozoa, yeasts, spores and pollen. Kauffmann (1948) tested the hæmagglutinating property of *Escherichia coli* against red cells of guinea-pig, horse, rabbit and human beings. According to him, hæmagglutinating and hæmolytic strains of *Escherichia coli* were more commonly seen among O-groups 4 and 6, and possessed higher pathogenicity. Investigations were, therefore, undertaken to carry out hæmagglutination tests with strains isolated from cases of urinary infection.

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TABLE II.
Hæmagglutination by Escherichia coli.

Number of strains.	Sheep	Rabbit	Rat	Human.	Chicken.	Guinea-pig.
5	—	—	—	—	—	—
21	—	—	—	—	—	—
34	—	—	—	—	—	—
15	—	—	—	—	—	—
12	—	—	—	—	—	—
2	—	—	—	—	—	—
6	—	—	—	—	—	—
69	—	—	—	—	—	—
101	—	—	—	—	—	—

— = Hæmagglutination present.
— = No hæmagglutination.

TABLE III.
O-group distribution of hæmagglutinating strains of Escherichia coli.

O-Group	Number of hæmagglutinating strains.
O-3	5
O-4	3
O-6	9
O-7	1
O-9	1
O-11	1
O-13	2
O-15	17
O-16	1
O-17	4
O-18	2
O-20	5
Unclassified	113

of red cells appeared to be widely different although the organism possessed the same O-antigen (Table IV):—

TABLE IV.
Hæmagglutination by strains of Escherichia coli belonging to O-group 6.

Strains number.	Sheep.	Rabbit.	Rat.	Human.	Chicken.	Guinea-pig.
E115	—	+	—	—	—	—
E124	—	—	—	+	—	—
E126	—	—	—	+	—	—
E127	—	—	—	+	—	—
E572	—	—	—	+	—	—
E578	+	—	—	+	—	—
E1653	—	—	—	+	—	—
E1733	—	—	—	+	—	—
E1797	—	—	—	—	+	—

+ = Hæmagglutination present.

— = No hæmagglutination.

AN EVALUATION OF THE MATSUBARA CARCINOREACTIN TEST.

BY

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[Received for publication, July 18, 1957.]

THE need for a reliable diagnostic test for cancer has been well realized not only because of the complicated nature of the disease but also due to the fact that its early diagnosis helps considerably in its successful treatment. Attempts to develop such a test could be traced to Fulci (1910) who showed qualitatively that a tumour tissue contains greater amount of lactic acid than its normal counterpart. Since then, several workers in different parts of the world have been engaged in developing a diagnostic test for cancer.

A review of the literature showed that as many as sixty-nine tests were proposed till 1950 as diagnostic aids to cancer. Of these, three depended upon morphological techniques, nine on immunological changes, and the rest on biochemical changes in stomach juice, blood and urine. Of the biochemical tests (Black, 1947; Black *et al.*, 1948; Winzler and Smith, 1948; Huggins *et al.*, 1949), based on changes in blood constituents, only four, viz. methylene blue-reduction test, heat-turbidity index, plasma-mucoprotein levels in blood and iodo-acetate index test were considered to be more dependable than others. However, Henry *et al.* (1951) later on showed that even these tests were not so reliable and specific as claimed originally. Although dependable biochemical tests for certain types of cancer, e.g. serum-acid phosphatase in metastasizing cancer of the prostate and serum alkaline phosphatase in bone tumours, are known, there is so far not a single reliable diagnostic test, chemical or otherwise, possessing a general applicability towards varied types of cancers. In view of this fact, it was considered desirable to evaluate the Matsubara carcino-reactin test, which was claimed by its inventor to be specific and of great value in the early diagnosis of any type of cancer. The work was carried out during the year 1954-55 (Technical Report of the Scientific Advisory Board, Indian Council of Medical Research, 1955).

The Matsubara test (1946 and 1951) depends on the immunobiological action of a polysaccharide compound isolated from cancer or placental tissue. In this test, a known amount of the solution of this compound in normal saline containing 0.5 per cent by volume of phenol is injected intradermally in healthy persons or cancer patients, and the reaction which appears as an erythema, is observed after four hours. According to Matsubara, the reaction was more pronounced in cancer patients (diameter of erythema, 20 mm. or more), than in healthy persons (diameter of erythema less than 12 mm.). His experimental results which were confirmed by Murata and Matsubara (1950) showed that the test was positive in 92.2 per cent of cancer cases and 4.3 per cent of the healthy subjects. This test was also positive in 98.3 per cent of pregnancy cases because of the resemblance between placental tissues and cancer tissues in growth, tissue respiration and glycolytic action. According to him, it is negative, in other pathological conditions, such as benign tumour, gastric ulcer, gastritis, cholecystitis and syphilis, which often manifest a false positive reaction to various cancer tests so far known. Thus, he claims that this test is specific and reliable in the early diagnosis of cancer and pregnancy, and often shows negative reaction at the later stage of the above cases.

It is the purpose of this paper to evaluate the utility of this test in the diagnosis of cancer.

MATERIAL AND TECHNIQUE.

The material for each test consisted of a vial containing 8 mg. of the polysaccharide and two 1-c.c. ampoules, one containing control fluid and the other solvent fluid for dissolving the substance. The entire material required in the present studies was supplied by Dr. Matsubara.

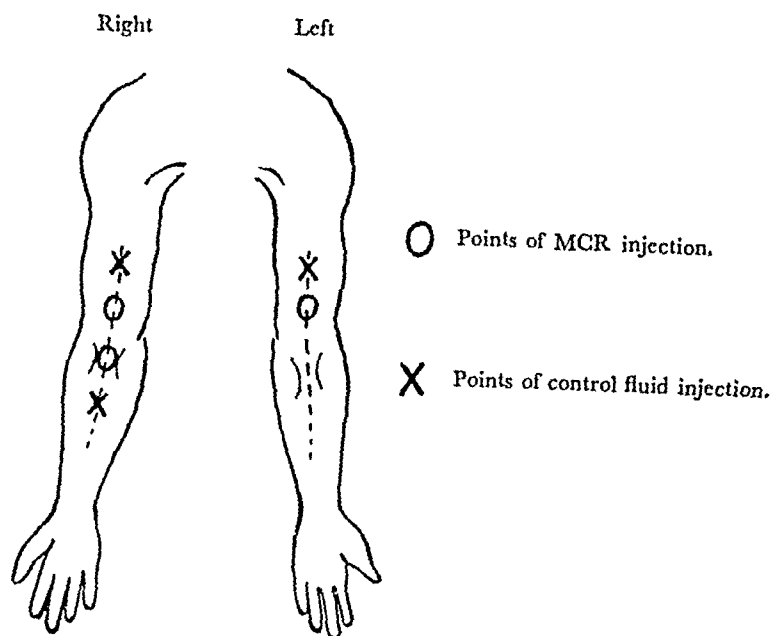
TECHNIQUE OF THE TEST.

0.2 c.c. of solution of the compound (8 mg. in 0.8 c.c. of the control fluid) is injected intradermally at three points each on the forearm and the upper arm, along the middle line of the flexor surface near the elbow joint. Then, 0.2 c.c. of control fluid is similarly injected at three points, each three cm. away and upwards along the line from the site of injection of the test fluid as shown in Text-figure. The measurement of the control and the test erythema are carried out four hours, after the injection.

These instructions were strictly followed and the test was run on 19 healthy subjects from Tata Memorial Hospital and the Indian Cancer Research Centre (11 males and 8 females) ranging in age from 25 to 45 years and 67 cancer patients who attended the Out-patient Department of the Tata Memorial Hospital. The measurement of the control and the test erythema were carried out by the same person in order to avoid personal error.

All the cancer patients selected for study were those in whom the histological confirmation of the disease was previously obtained and who had not undergone either x-ray or any other treatment for cancer. They had cancer at different sites; the distribution according to the sites is 46 (oropharyngeal region); 16 (cervix); 2 (rectum) and one each of leg, breast and vaginal wall.

TEXT-FIGURE.



RESULTS AND DISCUSSION.

Although Matsubara has based his conclusions on the diameters of the erythema in controls and cancer patients, we found it more appropriate to calculate the area of the erythema which were more or less elliptical. The area of the erythema was calculated by using the formula $A \text{ (area)} = \pi \left(\frac{a+b}{4} \right)^2$ where 'a' is the major axis and 'b' minor axis of the ellipse.

The results are summarized in Table I.

It was observed that in normal subjects the average area for the control erythema is 23.48 ± 2.66 sq. mm. and that for the test erythema is 137.6 ± 10.64 mm. According to the standards set by Matsubara, the test is positive if the area of the test erythema is more than 265 sq. mm. (i.e. diameter of erythema more than 18 to 19 mm.) and that of the control erythema is less than 80 sq. mm. (i.e. diameter of erythema less than 10 mm.). The test is indeterminate if the area of the control erythema exceeds 80 sq. mm. even though the test erythema may be more than 265 sq. mm. in area. In our studies, none of the normal subjects showed a false positive reaction and no case was come across which showed an indeterminate reaction. The test was positive in only 36 (53.7 per cent) of the 67 cancer cases studied; the remaining 31 cancer cases showed negative response to the test.

As an alternative method of evaluating the results, histograms were plotted for the normal subjects and the cancer patients as shown in the Graph. It can be seen from the Graph, that there is an overlapping between the values for normal and

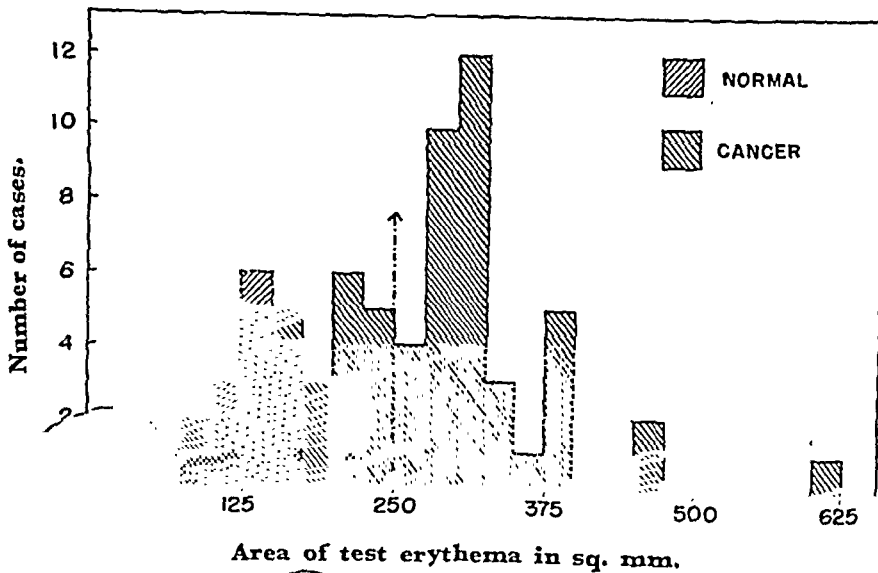
cancer cases up to an area of 225 sq. mm. of the test erythema. This indicated that all those cancer cases with the area of the test erythema exceeding 250 sq. mm. were positive to the test and that the test was not applicable to those cancer cases which lie in the region where the two histograms overlap each other. The results calculated on this basis showed that of 67 cancer cases studied the test was positive in 38 cases (56·6 per cent) and negative in others (43·4 per cent). This result was not materially different from that calculated on the basis of the standards prescribed by Matsubara.

TABLE I.

Summarizing results of the M.C.R. test.

Number.	Types of cases.	Number of cases.	M.C.R. TEST:	
			Positive.	Negative.
1.	Healthy adult controls ...	19	Nil	19 (100 per cent)
2.	Histologically proved cancer cases without clinical evidence of metastases.	52	29 (55·8 per cent)	23 (44·2 per cent)
3.	Histologically proved cancer cases with clinical evidence of advanced stage of the disease.	15	7 (46·7 per cent)	8 (53·3 per cent)
	Total ...	67	36 (53·7 per cent)	31 (46·3 per cent)
	Average age ...	...	47·2 years (33-65 years)	46·2 years (26-65 years)

GRAPH.



It will be seen from Table I that of the 67 cancer cases, 52 were without clinical evidence of metastases, while 15 had clinical evidence of advanced stage of the disease. Although the percentage of false negatives in the former group is less than that in the latter, still it is too high to allow the use of this test as a reliable index for cancer in its early stages. This also suggests that the response to the test does not show any significant relationship to the stage of the disease as claimed by Matsubara. It would be seen from Table II that squamous and epidermoid carcinomas formed about 95 per cent of the total cases studied. The test was positive in 21 of the 39 squamous carcinomas and in 12 of the 24 epidermoid carcinomas. Similarly, reference to Table III would show that the test was positive in 26 of the 46 cases of cancer of the oropharyngeal region and in 7 of the 16 cases of cancer of the cervix. This clearly indicates that the response to this test is independent of either the type or the site of carcinoma. It is also independent of the age of the patient as seen from Table I.

TABLE II.

Distribution of cases according to the types of carcinoma.

Type of cancer.	Number of cases.	REACTION TO M.C.P. TEST	
		Positive.	Negative.
1. Squamous carcinoma	39	21	18
2. Epidermoid carcinoma	24	12	12
3. Adenocarcinoma	1	1	0
4. Cylindroma	1	1	0
5. Alveolar carcinoma	1	1	0
6. Undetermined (Exfoliative cytological finding: Abnormal cells class III)	1	1	0
Total ...	67	36	31

TABLE III.

Distribution of cases according to the site of cancer.

Site of cancer.	Number of cases.	REACTION TO M.C.P. TEST	
		Positive.	Negative.
1. Oropharyngeal region	46	26	20
2. Cervix	16	7	9
3. Rectum	2	1	1
4. Vaginal wall	1	1	0
5. Leg	1	1	0
6. Breast	1	1	0
Total ...	67	36	31

The test under investigation, although technically simple and specific, does not fulfil the other important criteria of an ideal diagnostic test, viz. the high percentage (95 per cent) of true positives and the absence of any overlapping between the values for normals and those of diseased cases, suggested by Homburger (1950).

Youden (1950) proposed a new index for rating diagnostic tests. The formula for the index of performance is $J = \frac{ad-bc}{(a+b)(c+d)}$ where, of (a+b) diseased patients, 'a' are correctly diagnosed and 'b' are false negatives, and of (c+d) controls, 'd' are correctly reported and 'c' are false positives. The Youden's index calculated for this test using the above formula is 0.56, while for an ideal test it should be 0.95 to 1.0.

SUMMARY.

Matsubara carcino-reactin test was evaluated on 67 histologically proved cancer cases. The test was positive in only about 55 per cent of the cases studied, while the rest showed negative response. No false positive was observed. In our opinion the percentage of false negatives is too high to permit its use as a reliable index for cancer in its early stages. The diagnostic utility of the test has been discussed.

The authors express their sincere thanks to Dr. V. R. Khanolkar, Director, Indian Cancer Research Centre, Bombay, for his keen interest and the facilities offered and to Dr. L. D. Sanghvi, Statistician, Indian Cancer Research Centre for helpful suggestions.

The authors are also grateful to Dr. Matsubara for the supply of test material gratis and to the Surgeons and other staff of the Tata Memorial Hospital for their kind co-operation. Authors' thanks are also due to the Indian Council of Medical Research under whose auspices the work was carried out.

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BLOOD GLUTATHIONE AND ITS RELATION WITH HÆMO- GLOBIN AND RED BLOOD CELL COUNT IN TUBERCU- LOSIS, LEPROSY AND DIABETES.

Part II.

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[Received for publication, June 10, 1957.]

INTRODUCTION.

STUDY of the glutathione content of blood and its relation with hæmoglobin and red blood cell count in normal Indian subjects has already been published (Gokhale and Panse, 1955) by the present authors. As has already been indicated in the previous publication, this relation was being studied in certain diseased conditions, such as leprosy, tuberculosis and diabetes. The present paper deals with the investigations carried out on the above disease subjects. Similar studies had been carried out by Woodward and Fry (1932) in cancers, Ross and Hillary (1939) in leprosy, and Lazarow and Patterson (1948), and Illing, Gray and Lawrence (1951) in diabetes. The data, however, are not sufficiently adequate to show whether the cellular oxidation-reduction systems are affected in these diseased conditions. Therefore, the present study has been undertaken.

MATERIALS AND METHODS.

The bloods were collected from patients attending the Out-patients Departments of Tuberculosis, Leprosy and Medical Sections of the Sassoon Hospitals, Poona. The ages of the subjects were between 19 and 50 years in tuberculosis,

18 and 46 years in leprosy and 17 and 75 years in diabetes. Fasting bloods were collected in the morning in sterile penicillin bulbs containing dry potassium oxalate (2 mg. per c.c. of blood) and mixed thoroughly with the anticoagulant. The analysis of the blood samples was carried out immediately in most of the cases and when it was not possible to do so for about two hours, the blood was kept in the refrigerator.

The methods used for the determinations were as follows:—

(1) Glutathione, total and reduced (Woodward and Fry, *loc. cit.*); oxidized glutathione was obtained by the difference between the total and the reduced values.

(2) Red blood cell count was determined by use of a standard counting chamber (improved Neubaur) and standard diluting pipette.

(3) Haemoglobin by Sahli's method.

(4) Blood glucose by Folin and Wu (1920) method.

RESULTS.

The average values obtained for the red blood cell count, hæmoglobin percentage, total, reduced and oxidized glutathione and the four quotients, viz. total GSH/rbc, total GSH $\times$ Hb/rbc, R-GSH/rbc, R-GSH $\times$ Hb/rbc, of the one hundred and seven subjects of tuberculosis are given (Table I):—

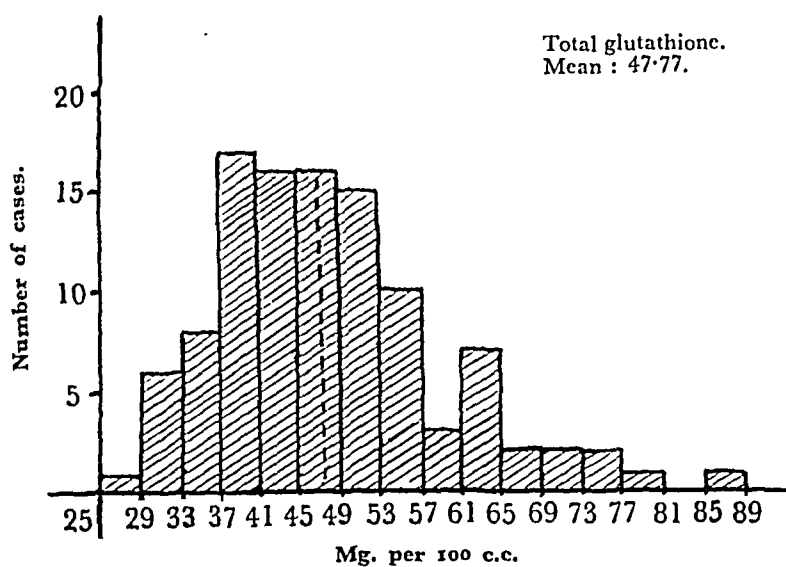
TABLE I.

Average values of rbc, Hb percentage, total, reduced and oxidized glutathione of 107 tuberculosis subjects.

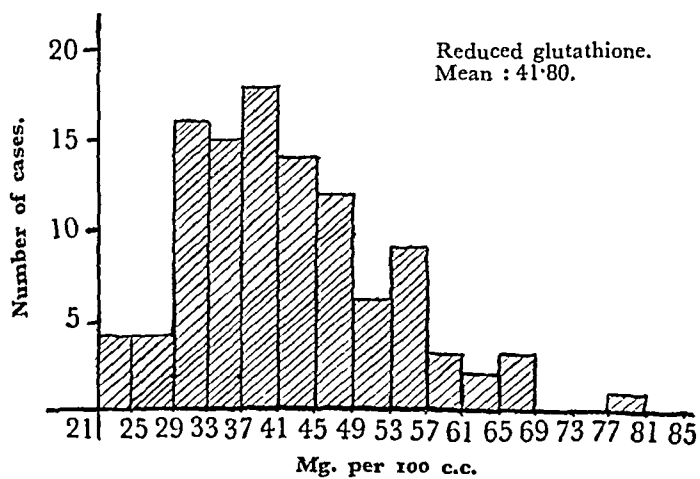
	Average.	Maximum.	Minimum.	Standard deviation, $\pm$.	Percentage within significant variation.	Coeff. of variation.	Standard error.
Red blood cell count, millions/c.mm.	3.97	5.26	2.46	0.723	...	...	...
Hæmoglobin, g. per cent	12.53	17.00	8.00	2.021	...	...	...
Total glutathione, mg. per cent	47.77	87.90	26.60	11.295	67	23.64	1.09
Reduced glutathione, mg. per cent	41.80	78.50	21.40	10.623	76	25.41	1.02
Oxidized glutathione, mg. per cent	6.02	16.60	0.80	2.623	84	43.58	0.25
Gabbe's quotient, GSH/rbc	12.37	25.00	7.20	3.525	...	...	...
Total GSH $\times$ Hb	...	...	...	...	...	...	...
rbc	152.10	262.5	86.70	34.974	...	...	...
Reduced GSH/rbc	10.81	22.36	6.08	3.200	...	...	...
Reduced GSH $\times$ Hb	...	...	...	...	...	...	...
rbc	131.50	234.8	62.62	34.70	...	...	...

The frequency distribution for total, reduced and oxidized glutathione is presented in Graphs 1, 2 and 3, respectively.

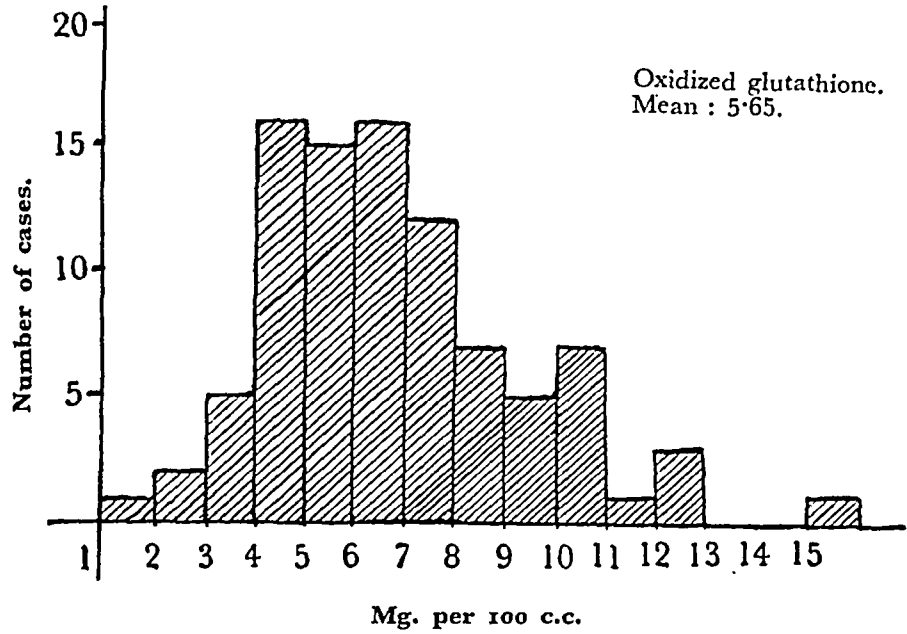
GRAPH 1.



GRAPH 2.



GRAPH. 6.



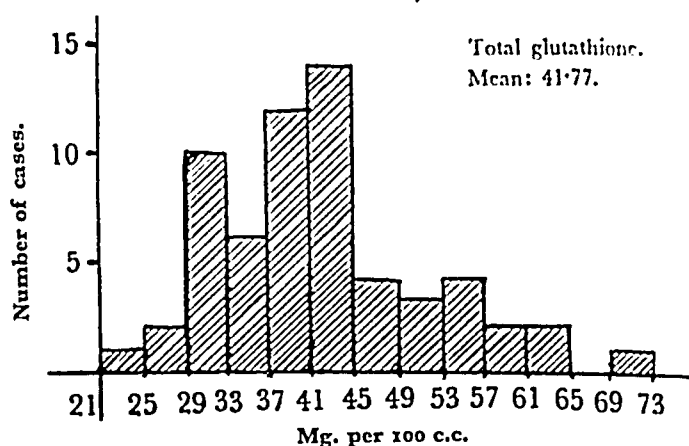
The average values obtained for the red blood cell count, hæmoglobin percentage, total, reduced and oxidized glutathione and the four quotients: total GSH/rbc, total GSH $\times$ Hb/rbc, reduced GSH/Hb, reduced GSH $\times$ Hb/rbc of sixty-one diabetic subjects are given in Table III.

TABLE III.
Average values of rbc, Hb percentage, total, reduced and oxidized glutathione of 61 diabetic subjects.

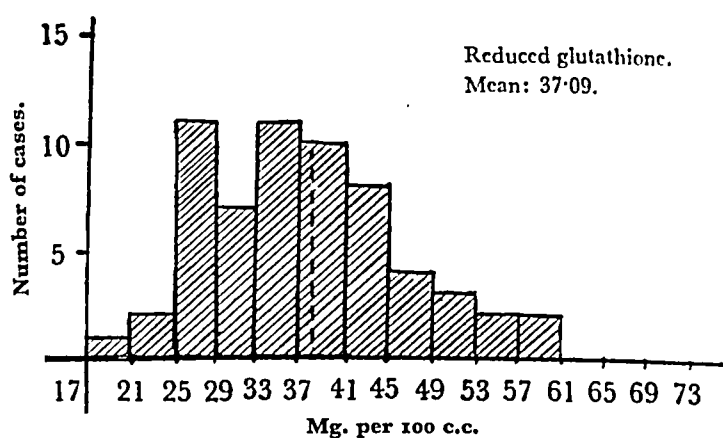
	Average.	Maxi- mum.	Mini- mum.	Standard devia- tion, ±.	Percent- age with in signi- ficant varia- tion.	Coeff. of varia- tion.	Stan- dard error.
Red blood cell count, millions c.mm.	4.20	5.27	2.92	0.60	...	...	...
Hæmoglobin, g. per cent ...	13.87	18.00	9.00	1.76	...	...	...
Total glutathione, mg. per cent ...	41.76	71.00	23.00	9.51	75	22.77	1.21
Reduced glutathione, mg. per cent ...	37.09	57.90	19.90	8.90	75	23.99	1.13
Oxidized glutathione, mg. per cent ...	4.71	12.40	0.50	2.53	77	53.87	0.32
Gabbe's quotient, GSH/rbc ...	9.99	17.68	6.23	2.04	...	...	...
Total GSH $\times$ Hb rbc ...	37.65	223.30	77.27	28.23	...	...	...
Reduced GSH/rbc ...	9.04	16.16	5.54	1.52	...	...	...
Reduced GSH $\times$ Hb rbc ...	121.94	185.84	62.59	26.06	...	...	...
Glucose ...	228.9	448.0	100.0	91.57	...	...	...

The frequency distribution for total, reduced and oxidized glutathione in diabetic subjects can be seen in Graphs 7, 8 and 9, respectively.

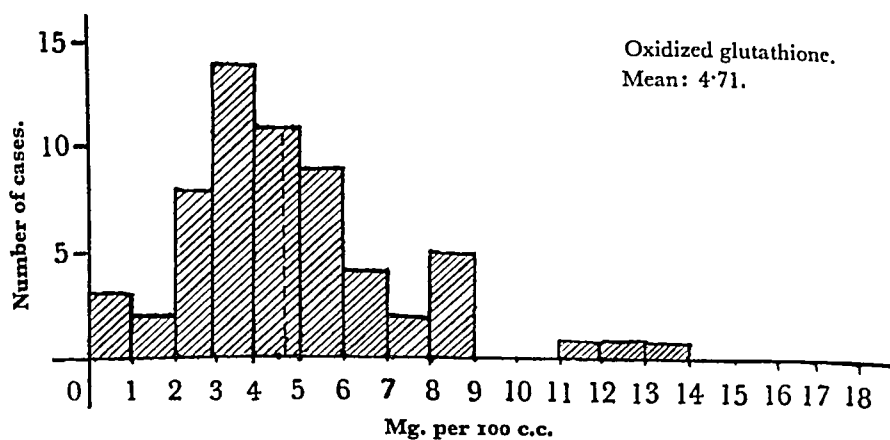
GRAPH 7.



GRAPH 8.



GRAPH 9.



Blood Glutathione and its Relation with Hemoglobin, etc.

TABLE IV.

Study of average values, difference in the means, standard error of the difference in the means in normal, tuberculosis, leprosy and diabetic subjects.

Study of average values, difference in
in the means in normal, tuberculosis, leprosy and diabetic subjects.

	NOR- MAL:	TUBERCULOSIS:			LEPROSY:			DIABETES:		
	Mean.	Mean.	Difference in the mean.	Standard error of difference in the mean.	Mean.	Difference in the mean.	Standard error of difference in the mean.	Mean.	Difference in the mean.	Standard error of difference in the mean.
1	2	3	4	5	6	7	8	9	10	11
I. Red blood cell count, millions/c.mm. ...	4.86	3.97	0.89	0.08	4.16	0.70	0.07	4.20	0.66	0.07
II. Hæmoglobin, g. per cent ...	14.57	12.53	2.04	0.26	13.06	1.50	0.25	13.87	0.70	0.28
III. Total GSH, mg. per cent ...	41.25	47.77	6.52	1.29	45.33	4.08	1.18	41.76	0.51	1.27
IV. Reduced GSH,mg.per cent ...	36.50	41.80	5.30	1.25	39.68	3.18	1.21	37.09	0.59	1.26
V. Oxidized GSH,mg.per cent ...	4.75	6.02	1.27	0.32	5.65	0.90	0.31	4.71	0.40	0.35
VI. Glucose, mg. per cent ...	98.24	99.60	8.65	...	81.60	16.67	...	228.94	130.70	...
VII. <u>Total GSH</u> rbc ...	8.61	12.37	3.76	0.38	11.08	2.47	0.31	9.99	1.38	0.27
VIII. <u>Total GSH×Hb</u> rbc ...	120.04	162.08	42.04	1.30	151.18	31.14	3.81	137.65	17.61	4.06
IX. <u>Reduced GSH</u> rbc ...	7.67	10.81	3.14	0.35	9.69	2.02	0.32	9.04	1.37	0.25
X. <u>Reduced GSH×Hb</u> rbc ...	110.30	131.48	21.18	4.11	124.25	13.95	3.72	121.95	11.65	3.58

DISCUSSION.

DISCUSSION.

In Table IV are presented the means, difference in the means for the sake of comparison by statistical examination of data obtained in the cases of diseased conditions with that of normal subjects. The values of the normal subjects have already been published (Gokhale and Panse, *loc. cit.*) but they are given in the present paper wherever required.

The frequency distribution for total, reduced and oxidized glutathione in diabetic subjects can be seen in Graphs 7, 8 and 9, respectively.

Tuberculosis.—The subjects of this study were suffering from pulmonary tuberculosis. They were not hospitalized and used to attend the Out-patients Clinical Departments. The results embodied in Table IV show that the average values for one hundred and seven subjects, of glutathione, viz. total 47.77 mg., reduced 41.80 mg. and oxidized 6.02 mg. per 100 c.c. of blood are more than the corresponding average values obtained for one hundred and two normal subjects studied, viz., total 41.25 mg., reduced 36.50 mg., and oxidized 4.75 mg. per 100 c.c. These differences have been found to be statistically significant. The only data by the other workers that could be obtained by us from the literature was that of only one tuberculosis subject studied by Caren and Carne (1951). They found

the blood glutathione level to be normal, viz. 33.7 mg. per cent in their case of pulmonary tuberculosis. The other relations, viz., total GSH/rbc, total GSH $\times$ Hb/rbc, reduced GSH/rbc, reduced GSH $\times$ Hb/rbc between the glutathione and the rbc and the haemoglobin are also significantly increased in tuberculosis. This is, however, due to the glutathione being significantly increased.

Leprosy.—The subjects of this study were suffering from all types of leprosy, such as nodular, tubercular and showed anaesthetic patches all over the body. They were not hospitalized but used to attend the leprosy clinics. The results given in Table IV show that the average values for the ninety-one subjects studied, of glutathione, viz. total 45.33 mg. reduced 39.68 mg., and oxidized 5.65 mg. per 100 c.c. of blood are more than the corresponding average figures obtained for one hundred and two normal subjects as given above. These differences have been found to be statistically significant. The only data available were those of Ross and Hilary (1939) who state that 'In 96 leprosy subjects blood contents of reduced glutathione was essentially normal, but in 23 of the cases, oxidized glutathione was increased'.

Diabetes.—The subjects of this study were diabetics whose urine showed reduction with Benedict's solution. The bloods were essentially collected on fasting stomach. The results given in Table IV show that the average values for the sixty-one subjects studied, of the glutathione, viz. total 41.76 mg., reduced 37.09 mg., and oxidized 4.71 mg. per 100 c.c. of blood, do not show any significant difference in the normal subjects and the difference obtained so far is not statistically significant. Out of the sixty-one diabetic subjects of the present study, six subjects had fasting blood sugar over 350 mg. per cent and the average glutathione values for these were: Total GSH 37.83 mg., and reduced GSH 34.03 mg. per 100 c.c. These are lower than the normal average values. There are fifteen diabetic subjects having fasting blood sugar level over 300 mg. per cent in this series. Two of them gave very high values for total and reduced GSH content, viz. 58.0 and 71.1 mg. per cent. If these two subjects are not taken into account, the rest thirteen diabetic subjects showed average total and reduced GSH as 35.95 mg. and 32.93 mg. per cent. These values are lower than the normal average values obtained previously. Similarly, there are twenty diabetics having fasting blood sugar level over 250 mg. per cent in our series. Three of these gave for their total glutathione content very high values, viz. 58.0, and 60.5 mg. per cent. If these values are not considered, the rest seventeen subjects gave the following average values: Total GSH 36.70 mg. and reduced GSH 31.75 mg. per 100 c.c.

Caren and Carne (*loc. cit.*) found for their 42 diabetic subjects, fasting blood glutathione levels within the normal range, viz. 25 mg. to 41 mg. per 100 c.c. They also state that no correlation could be obtained between the blood sugar and blood glutathione. Stock and Currence (1950) have studied 14 subjects and obtained reduced glutathione values between 26 and 42 mg. per 100 c.c. Dogliotti and Melloni (1935), and Platt (1931) found no change in the fasting blood glutathione levels in the diabetic subjects. However, Verela, Apolo and Villar (1930), and Companacci (1930) have reported slightly lower values in diabetes. Illing, Gray and Lawrence (*loc. cit.*) studied 20 normal individuals, 18 diabetics without ketosis, and 21 diabetics having heavy ketosis. They state that a significant reduction in GSH was found in diabetics with severe ketosis. Their average figures

were: Normal:Total 43.0, reduced 33.5 mg. All diabetics—total GSH 39.1, reduced 30.1 mg., diabetics without ketosis—total GSH 41.1, reduced 32.1 mg. and diabetics with ketosis—total GSH 37.5, reduced 28.5 mg. per 100 c.c. of blood.

The following may be quoted from 'Glutathione—A symposium (1954)', "Although many investigators have reported a decreased concentration of GSH in the blood and the tissues of human diabetic subjects, this has not been a constant finding". Binkley *et al.* (1950) have obtained lower glutathione values in diabetes. But it should be noted that they have used a different technique and the findings have been based on a very small number of cases, namely five normals and ten diabetics. Glutathione values obtained in the present study and those obtained by the other workers are embodied in Table V for the purpose of comparison:—

TABLE V.

Worker.	Year.	Number of cases.	REDUCED GLUTATHIONE VALUES:	
			Normal.	Diabetes.
1. Verla, Apolo and Vilar	...	1930	...	Lower values than normal.
2. Campanacci	...	1930	...	Lower values than normal.
3. Platt	...	1930	...	Same values as in normal.
4. Dogliotti and Melloni	...	1935	...	Same values as in normal.
5. Binkley <i>et al.</i>	...	1950	10	16.9±1.2
6. Caren and Carne	...	1951	42	25 to 41
7. Stock and Currence	...	1950	14	26 to 42
8. Illing, Gray and Lawrence	...	1951	39	33.5
9. Present study	...	1957	61	36.50
			6	Blood sugar > 350 mg.
			13	Blood sugar > 300 mg.
			17	Blood sugar > 250 mg.

From the above data, it may be observed that one is not in a position to say definitely whether the blood glutathione values are reduced in diabetes.

The average values obtained for the four quotients, viz. total GSH/rbc, total GSH×Hb/rbc, reduced GSH/rbc, $\frac{\text{Reduced GSH} \times \text{Hb}}{\text{rbc}}$ in the diabetic subjects are 9.99, 137.65, 9.04, 121.94, respectively, as given in Table III.

These values are more than the values for normal subjects obtained previously and these are significant as shown in the Table IV.

SUMMARY.

(1) Bloods of one hundred and seven subjects of tuberculosis, ninety-one of leprosy and sixty-one of diabetes were studied for the following investigations: Glutathione: total, reduced, oxidized, red blood cells count and hæmoglobin.

(2) The average values are as follows:—

(a) *One hundred and seven tuberculosis subjects.*—

Total GSH	...	47.77 mg. per 100 c.c.
Reduced GSH	...	41.80 " " "
Oxidized GSH	...	6.02 " " "
Red blood cell count	...	3.97 millions/c.mm.
Hæmoglobin	...	12.5 per 100 c.c.

(b) *Ninety-one leprosy subjects.*—

Total GSH	...	45.33 mg. per 100 c.c.
Reduced GSH	...	39.68 " " "
Oxidized GSH	...	5.65 " " "
Red blood cell count	...	4.16 millions/c.mm.
Hæmoglobin	...	13.06 g. per 100 c.c.

(c) *Sixty-one diabetic subjects.*—

Total GSH	...	41.76 mg. per 100 c.c.
Reduced GSH	...	37.09 " " "
Oxidized GSH	...	4.71 " " "
Red blood cell count	...	4.20 millions/ c.mm.
Hæmoglobin	...	13.87 g. per 100 c.c.

(3) The following relationships have been calculated from the values of glutathione, rbc, and hæmoglobin. Their average values are:—

	Tuberculosis.	Leprosy.	Diabetes.
Total GSH/rbc	12.37	11.08	9.99
Total GSH × Hb./rbc	162.08	142.17	137.65
Reduced GSH/r.b.c.	10.81	9.69	9.04
Reduced GSH × Hb/rbc.	131.48	124.25	121.95

(4) The blood glutathione average values were found to be higher in tuberculosis (total 47.77, reduced 41.08 mg.) than in normal subjects (total 41.25 mg., reduced 36.50 mg.).

(5) The blood glutathione average values were found to be higher in leprosy (total 45.33, reduced 39.68 mg.) than those in normal subjects.

(6) In all diabetic subjects the average values of total GSH and reduced GSH do not show any significant difference from those of normal subjects. But when these diabetic subjects were classified into three groups having blood sugar levels over 350 mg., 300 mg., and 250 mg., the following interesting results were obtained. The six selected subjects having blood sugar level over 350 mg. gave the average values for reduced GSH as 34.03 mg. as against 36.50 mg. of reduced GSH in normal subjects. Similarly, thirteen selected subjects having blood sugar level over 300 mg. gave 32.93 mg. of reduced GSH which is less than normal. As also seventeen selected diabetic subjects having blood sugar level more than 250 mg. showed their reduced GSH values as 31.75 mg. which is less than normal. From the results obtained in this study it is very difficult to state definitely whether the blood glutathione content is decreased in diabetes.

The authors express their thanks to the Principal and the Professor of Pathology, B. J. Medical College, Poona, and to the Civil Surgeon, Sassoon Hospitals, Poona, for giving the Laboratory facilities and to the Honorary Medical Officers, Tuberculosis, Leprosy, and Medical Units of the Hospitals for making available the subjects for the study. They are also thankful to Mr. S. K. Karandikar, M.Sc., for his statistical assistance in this work.

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A MODIFIED GELATIN AS A PLASMA EXPANDER.

Part I.

EXPERIMENTS ON HÆMORRHAGED DOGS.

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A NEW modified gelatin suitable for intravenous injection as a plasma expander was prepared at the National Chemical Laboratory. The trials on animals were carried out at the Armed Forces Medical College. Dextran (Benger Laboratories Ltd., U.K.) and modified fluid gelatin (Charles B. Knox Co., U.S.A.) were tried under identical conditions for comparison. The results of these experiments are reported in this paper.

The term 'plasma expander' is considered more suitable than any other term for fluids injected to increase the plasma volume, and for brevity 'expander' is used in the text.

MATERIAL AND METHODS.

The gelatin was extracted from bones obtained from slaughter houses; and the yield from one kilogram of bones was nearly 1 litre of a 6 per cent solution. The trial batches were prepared on laboratory scale; therefore, the quantity was small, and the experiments with every batch were restricted to 5 dogs. The concentration of early batches was 8 per cent, but the physical properties were gradually improved, the results were assessed on dogs, and ultimately the concentration was reduced to 6 per cent with improvement in quality. Attempts are being made to improve it still further and reduce the concentration.

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The sixth batch (MG-6) was an 8 per cent solution, relative viscosity 1.56 and gelling temperature 4°C. This batch was used to test toxicity, sensitizing and antigenic property, as well as for experiments on the rate of excretion in dogs. The next batch MG-7, was a 6 per cent solution, relative viscosity 2.2, and gelling temperature 10°C.; the experiments on hæmorrhaged dogs and the effects on antibody formation were carried out with this batch.

Toxicity.—Two rabbits were given 20 c.c./kg. body-weight, intraperitoneally, after withdrawing an equal quantity of blood; and 4 guinea-pigs were given 4 c.c./100 g. body-weight, intraperitoneally. All animals were observed for one month.

Sensitizing property.—Sensitizing doses were given on 3 successive days. The single dose for rabbit was 10 c.c., intravenously, or 0.5 c.c., subcutaneously; for guinea-pigs 3.0 c.c., intraperitoneally. Twenty-one days after the last dose, twice the quantity of a single dose was injected by the same route as the sensitizing dose.

Antigenic property.—Rabbits, guinea-pigs and white rats were injected biweekly 5 c.c., 1.0 c.c. and 0.5 c.c., respectively, by the subcutaneous route for 3 weeks. Ten days after the last injection the serum was examined by precipitin test by the Boyd (1947) technique.

Sera of 154 healthy persons were examined for complement fixing antibodies against MG-7 and dextran.

Effect on antibody formation.—A virus, a bacteria and sheep cells were selected to study the effect of intravenous infusion of MG-7 on the formation of antibodies.

1. *Influenza A-FMI strain, allantoic fluid containing 750 CCA units per c.c.*—Two doses of 3 c.c. and 9 c.c. were injected intravenously into fowls at an interval of one week along with 25 c.c. of MG-7.

2. *Heat-killed suspension of S. typhosum containing 250 million organisms per c.c.*—Rabbits were given 7 injections subcutaneously at intervals of 4 days starting with 0.25 c.c., and increasing after every two doses by 0.25 c.c. to a maximum of 1.0 c.c.; and 25 c.c. of MG-7 was given simultaneously with each injection by the intravenous route.

3. *Ten per cent suspension of sheep cells in normal saline.* Three injections of 5 c.c. each were given intravenously to rabbits at intervals of one week along with 50 c.c. of MG-7. All animals were bled before starting the injections and 10 days after the last injection to test for antibodies.

Coagulation time.—Coagulation time was measured by the capillary method (Wintrobe, 1947).

Erythrocyte sedimentation rate (ESR).—ESR was estimated in Wintrobe tubes. A platform with a glass top supported on vertically adjustable knobs was improvised. The level of the glass plate was corrected in two planes with a spirit level. The tubes were fixed on the platform with plasticine and were aligned with the vertical axis of a broad based right-angled triangle.

Estimation of gelatin and dextran in blood and urine was carried out by the method of Hoffman and Kozoll (1946) and Metcalf and Rouselot (1952), respectively.

Excretion of gelatin.—The experiment was carried out on 3 dogs. Under nembutal anaesthesia the femoral artery and vein on the same side were exposed. The blood volume was determined. Half of the blood was withdrawn through the artery and an equal volume of gelatin was injected through the vein. The skin was sutured. The dog was kept in a metabolic cage till the termination of the experiment. The gelatin present in the blood and the amount excreted in the urine in 24 hours was determined daily.

Water-holding capacity (WHC) of the expander.—It was reported by Wiggers (1950) that in non-fatal haemorrhage regeneration of plasma proteins does not occur for at least 6 hours; therefore, it was presumed that any change observed in plasma protein concentration at the end of 4 hours after infusion was due to dilution with the expander and the dilution was calculated by the following formula:

$$c = v \left(1 - \frac{p}{P} \right)$$

The symbols c , v , p , P are the expander retained in the blood stream, plasma volume at the end of 4 hours, plasma protein concentration at the end of 4 hours, and the initial plasma protein concentration, respectively. The water-holding capacity of the residual expander in the blood was calculated from the quantity of the fluid and the solid component of the expander retained in circulation.

Plasma volume, blood volume and fatal re-bleeding volume indices.—Plasma volume index (PVI) was the volume of the plasma at the end of 4 hours, expressed as percentage of the expected plasma volume. The expected plasma volume was the sum of the plasma left in circulation after haemorrhage plus the quantity of the expander introduced. The blood volume index was the blood volume at the end of 4 hours expressed as percentage of the initial blood volume.

At the end of the experiment the dog was bled to death and the quantity removed was calculated as percentage of the blood volume estimated just before bleeding, and was labelled as fatal re-bleeding volume index (FRVI). It broadly indicates the physiological state of the animal. For controls, the fatal bleeding volume of 6 healthy dogs was determined by measuring the blood volume and then bleeding them to death.

The injection of the expander after a single massive haemorrhage and further observations were carried out according to the technique given by Singh and Kalra (1956).

RESULTS.

MG-6 was non-toxic, non-antigenic and did not sensitize the animals. All human sera gave a negative complement-fixation test with MG-7; but one fixed complement in the presence of dextran in a dilution of 1:8 (++) , but when the individual was given 0.1 c.c. intradermally there was no local or generalized reaction. MG-7 did not interfere with the formation of antibodies (Table I).

TABLE I.

Effect of gelatin on the formation of antibodies.

Animal.	Antigen.	ANTIBODY TITRE I:	
		With gelatin	Without gelatin.
Rabbits	10 per cent washed sheep cells	1,000	8,000
		1,200	1,000
		1,000	
		8,000	
Rabbits	<i>S. typhosum</i> vaccine	7,000	8,000
		20,480	
Fowls	Influenza-FMI	512	2,000
		1,012	4,000
		8,000	

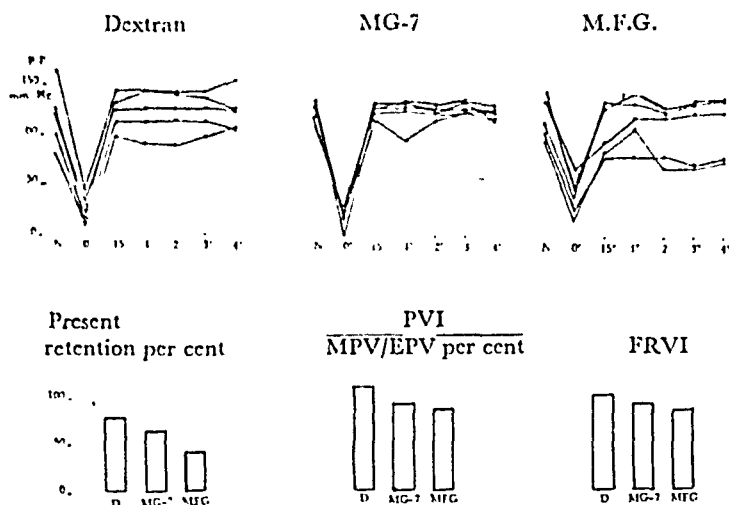
Survival.—When a dog survived the duration of the experiment (4 hours) it was recorded as survived. It was previously reported (Singh and Kalra, *loc. cit.*) that when half of the blood was withdrawn and no replacement fluid was given 3 dogs out of 10 survived. If blood was replaced by saline, 4 dogs out of 6 survived. The expanders tried in this series saved all dogs.

Blood pressure.—After hæmorrhage the blood pressure came down to an average of 33 mm. Hg. Dextran restored the pressure to normal within 15 minutes in 4 dogs and maintained it at that level. In the 5th dog the blood pressure remained 18 mm. Hg. below the original level throughout the experiment. MG-7 restored the blood pressure of all dogs to normal within 15 minutes; later in 3 dogs it stayed at the original level and in 2 dogs it gradually dropped by 16 and 18 mm. Hg. below the initial level, by the end of 4 hours. Modified fluid gelatin (MFG) restored the blood pressure slowly. In 3 dogs it gradually reached close to the original level and stayed at that level; in 2 dogs, after the initial rise there was gradual fall and by the end of 4 hours it was 16 and 38 mm. Hg. below their normal level (*vide* Graph).

Plasma volume and blood volume.—The water-holding capacity of dextran was 17.0 ± 4.2 c.c./g. and only 25 per cent of the solid was eliminated in 4 hours. The PVI was 104 and the blood volume at the end of 4 hours was higher than original. MG-7 had a lower WHC (14.3 ± 2.5 c.c./g.) and 37 per cent of the solid was eliminated in 4 hours; the PVI was 89.7 per cent and the average decrease in blood volume was 7.6 per cent. The WHC of MFG was very high 39.0 ± 8.4 c.c./g., therefore, in spite of the fact that it was a 3 per cent solution and nearly 60 per cent of the solid was eliminated in 4 hours, the PVI was 82.

Non-protein nitrogen and sugar in the blood.—The total NPN in the blood increased in half of the dogs and the average increase was 13 ± 6 per cent only. Hyperglycemia followed in one-third of the dogs and the increase was limited to 12 ± 8 per cent. The changes were of little significance and were the same with every expander.

GRAPH.



Erythrocyte sedimentation rate (ESR).—When the ESR was initially high, it was increased still further by all expanders, but if it was normal in the beginning the change in the case of every expander was erratic. In 13 dogs the ESR was initially normal (0 to 1 mm.), and after infusion of an expander there was marked increase in 7 and no change in 6 (Table III). This phenomenon is being investigated further.

Clotting time.—The clotting time of 41 dogs varied from $1\frac{1}{2}$ to $5\frac{1}{2}$ minutes, and was not altered by any expander.

Excretion rate of gelatin.—In 11 dogs, 50 ± 9 per cent of the gelatin administered was present in circulation at the end of 4 hours. In another set of 3 dogs, who received the same batch of gelatin, none was present in circulation at the end of 4 days; and the 24-hour sample of urine collected on the 4th day was also free of gelatin in all animals. There were individual variations in the rate of excretion but the final results were identical. Daily estimations of gelatin excreted in the urine could be carried out on 2 dogs only due to an accident, and both of

TABLE II.

Changes in plasma volume, blood volume, fatal re-bleeding volume indices and other values with standard error.

	Dextran.	MG-7.	MFG.
Plasma volume index ...	104 ± 6.26	89.7 ± 5	82 ± 2.23
Blood volume index ...	111 ± 6.7	93 ± 6.26	86 ± 8.05
FRVI ...	48.6 ± 2.28	42.5 ± 4.3	37.6 ± 2.81
Retention of the { Solid, per cent	79.2 ± 5.54	63.3 ± 1.97	41.2 ± 2.15
expander { Fluid, per cent	72 ± 5.37	52.3 ± 5.0	48.4 ± 6.7
Water-holding capacity ...	17.0 ± 1.88	14.3 ± 1.12	39.0 ± 3.80

them individually excreted 85 per cent of the gelatin in the urine. The fate of the rest of gelatin retained in the body was not determined.

FRVI.—The fatal bleeding volume index (FBVI) of 6 healthy dogs was 58.2 ± 8.3 . The FRVI of the expanders was in the following order: Dextran 48.4 ± 5.2 , MG-7 42.5 ± 9.6 , MFG 37.6 ± 6.3 .

TABLE III.

ESR of individual dogs before and after injecting an expander.

DEXTRAN.		ESR IN MM.			
		MG-7.		MFG.	
		0 hour.	4 hours.	0 hour.	4 hours.
26	74	1.5	52	55	74
0	26	0.5	22	0	55
0.5	59	0.5	10	0	45
1	74	0.5	2	0	0
0	0	0.5	1	0	0

TABLE IV.

Percentage of gelatin (MG-6) in blood and urine, after removal of half of the blood and replacement with an equal quantity of MG-6.

Dog number.	Specimen.	INTERVAL IN DAYS:					Total excretion.
		1st.	2nd.	3rd.	4th.	5th.	
1 }	Blood	45.1	29.2	6.4	0	0	85.1
	Urine	41.5	14.5	7.9	21.2	0	
2 }	Blood	33.1	8.7	n.d.	0	0	85.1
	Urine	43.9	28.7	12.2	0.3	0	
3 }	Blood	32.1	16.1	0	0	0	
	Urine	n.d.	17.8	2.8	0.7	0	

DISCUSSION.

The results were statistically analysed according to Snedecor (1946) method. Values of 't' greater than 2.8 are considered significant. Differences between dextran and MG-7 with respect to the maintenance of blood pressure, plasma volume index, blood volume index, fatal re-bleeding volume index and water-holding capacity appear to be within the limits of experimental error. MFG, on the other hand, has shown a significantly higher water-holding capacity than both dextran and MG-7. When compared with MFG, however, dextran gave significantly higher values for plasma volume, blood volume and fatal re-bleeding volume indices.

Dextrans have certain side effects. Kabat and Berg (1952) have mentioned that dextrans may produce symptoms of urticaria, angioneurotic oedema and asthma. Hehre *et al.* (1952) reported that dextrans cross react with antisera of *Pneumococci* of type 2, 12, 20, certain *Streptococci*, typhoid bacteria and a number of other *Salmonella*. To observe whether T.A.B. vaccination will produce sensitivity to dextran, the sera for complement-fixation test were collected three weeks after primary vaccination of recruits with two doses of 0.25 c.c. and 0.5 c.c. The only individual that gave a positive reaction was also positive before, as well as after vaccination in the same titre, and there was no allergic reaction on intradermal injection of 0.1 c.c. Most probably, such defects have been considerably eliminated from the recent preparations. In dogs the capillary permeability was affected by dextran. The blood continued to ooze from cuts in the skin and other tissues, and even from needle punctures in the skin. On post mortem examination there was free fluid containing dextran in the peritoneal cavity.

The modified fluid gelatin (Knox. Co.) was not as good as the other expanders, but its concentration was half than that of MG-7 or dextran although the water-holding capacity c.c./g. was far greater.

The fluidity of MG-7 was within the normal limits of the relative viscosity of human plasma. It had no side effects, the capillary permeability was not affected, it had no toxic, allergic or antigenic property and did not affect the formation of antibodies. The gelling temperature was 10°C., therefore, it can be used in most of the climates without pre-warming to make it fluid. Storage at 37°C. for 1 year made no change in physical properties, and stability over longer periods at different temperatures is being studied. It fulfilled most of the requirements of a plasma expander and is considered suitable for intravenous infusion.

SUMMARY.

Experiments on the biological evaluation of a new modified gelatin are described. The relative viscosity of the gelatin was 2.2, and gelling temperature 10°C. It had no toxic, allergic or antigenic property and maintained blood pressure and blood volume of hæmorrhaged dogs.

The manufacture of this gelatin was initiated by Dr. M. Damodaran, formerly Head of the Division of Biochemistry of the National Chemical Laboratory, Poona. The authors are grateful to Professor G. I. Finch, Director of the National Chemical Laboratory and Surg. Captain B. L. Taneja, formerly Professor of Pathology at the Armed Forces Medical College, for their help and suggestions.

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MODIFIED GELATIN AS A PLASMA EXPANDER.

Part II.

EXPERIMENTS ON SPLENECTOMIZED DOGS.

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REEVE *et al.* (1953) reported that in barbutorized dogs the spleen is greatly dilated and the degree of filling of the spleen with the dye influences estimates of blood volume by this method. If the diffusion of the dye in the spleen is defective the estimation of blood volume will be lower than the actual. Wiggers (1950) stated that during massive hæmorrhage there is prompt and marked contraction of the spleen, and from estimation of changes in spleen volume in dogs during bleeding he concluded that 100 c.c. to 200 c.c. blood is added by the spleen to the circulating volume. In our experiments (Kalra *et al.*, 1956), nembutal was used for anæsthesia, and if the estimations of blood volume were lower than actual, it is possible that although fifty per cent of the estimated blood was removed, the quantity was really less than half. The final result, also, was a combined contribution of the expander and an unknown quantity of blood pushed into circulation by the spleen. Moreover, the dogs were not shocked and were not allowed to stay in the hypotensive state for more than 5 to 10 minutes, the time taken to measure the blood taken out and the infusion of the expander.

To increase the accuracy of the estimation of the blood volume by the dye-method and to rule out the addition of an unknown quantity of blood into circulation, a further series of experiments were carried out with MG-7 on splenectomized dogs.

The combined operation of splenectomy and massive hæmorrhage produced a variable degree of shock and proved fatal to 2 dogs out of 14, before any transfusion

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could be given. Altogether, the efficacy of the gelatin was put to a more rigorous test as compared to the experiments already reported (Kalra *et al.*, 1958)

TECHNIQUES.

The spleen was exposed by a lateral incision, the blood vessels were ligatured and the organ excised. After splenectomy the estimation of blood volume by the Evan's blue dye method took one hour or more and during this interval the blood pressure became stable.

The fatal bleeding volume (FBV) of normal dogs was 58 per cent of the total blood and removal of half of the blood did not lead to fatality if the hypotensive state was not prolonged. After excision of the spleen the average FBV of 8 dogs was 47.4 ± 4.9 per cent; therefore, it was not possible to remove half of the blood from every dog. The standard of massive hæmorrhage was altered and the new standard was fixed, to bleed the dog till the flow of the blood was reduced from continuous stream to separate drops. On an average, 40.6 ± 6.3 per cent of the whole blood was removed, its proportion to the FBV was 85 per cent, the same as in previous dogs whose spleen had not been removed.

In 9 dogs, Ca, K, and Mg., in the blood was estimated before hæmorrhage, and at the end of 4 hours after infusion of the expander. The deficiency of K and Mg. had been made up, but the loss of Ca was not restored to normal in 6 dogs.

Two series of experiments on splenectomized dogs were carried out. In one series, 6 dogs were injected with MG-7 in normal saline, and in the second series 6 dogs were injected with MG-7 dissolved in a balanced salt solution containing CaCl_2 0.025 per cent, KCl 0.042 per cent and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 0.0005 per cent in addition to NaCl .

RESULTS.

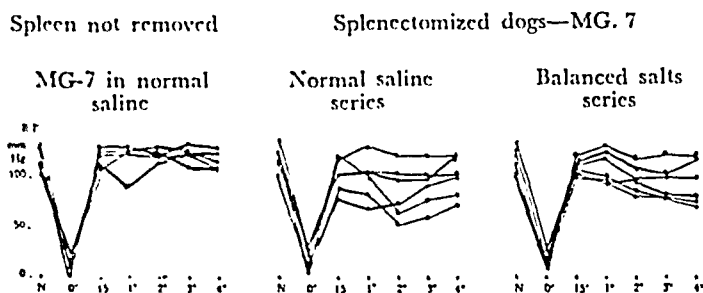
All dogs survived after transfusion. There was no change in the total red cells in circulation, and it is presumed that no blood was added to the circulating volume from any source.

After bleeding, the blood pressure came down to an average of 22 mm.Hg. as compared to 33 mm.Hg. in the previous series. In the normal saline series the blood pressure returned to normal within 15 minutes in 3 dogs and remained at that level for 4 hours. In the others it was 72 ± 11 per cent of the original level at the end of 4 hours.

In the balanced salt series the blood pressure returned to normal within 15 minutes after transfusion in 5 dogs and remained at that level for one hour; subsequently, the advantage was lost in 3 dogs. At the end of 4 hours it was at the original level in 2 dogs and in 4 dogs it was 69 ± 4 per cent of the initial level (*vide* Graph). Two dogs, whose blood pressure came down after one hour, were given amytal sodium for anæsthesia instead of nembutal. A total of 75 mg./kg. had to be given to keep the dogs under anæsthesia for 4 hours as compared to 35 to 45 mg./kg. of nembutal. The respiratory rate was markedly increased on administration of maintenance dosage after the first 40 mg./kg. It was later

detected that the batch of amytal sodium was toxic, and probably, it had contributed to the fall in blood pressure after one hour. In one of these animals the non-protein nitrogen was exceptionally high before hæmorrhage.

GRAPH.



There was no difference in the retention of the expander, maintenance of blood volume or FRVI in the two series.

The water-holding capacity of gelatin in splenectomized dogs was greater than in dogs whose spleen had not been removed, this was in all likelihood due to more accurate estimation of blood volume in the former. The non-protein nitrogen in the blood was significantly lower in the balanced salt series as compared to the normal saline series with the exception of one dog mentioned above.

TABLE I.

Results of MG-7, in normal saline and in balanced salt solution.

				Normal saline series.	Balanced salt series.
Plasma volume index	...	...	...	97.0±4.8	95.0±7.6
Blood volume index	...	...	...	99.0±3.4	97.0±8.6
WHC c.c./g.	...	...	...	18.6±2.4	19.5±5.1
FRVI	...	...	...	37.5±4.5	38.6±7.4
NPN mg. per cent (increase)	...	...	...	15.0±9.4	6.4±3.0

Effect on erythrocyte sedimentation rate (ESR).—In two dogs the ESR was initially high and increased still further on injecting gelatin. In 10 dogs the ESR was initially normal, in 5 it increased after injecting gelatin and in 5 it remained unaltered. *In vitro* experiments were carried out with the bloods by mixing different proportions of gelatin and equal parts of dextran to whole citrated blood and to 20 per cent suspension of washed cells in saline. The mixtures were incubated for half an hour at 37° C., and were frequently shaken during this period, before the test was put up. The effect on whole blood and on washed cells was the same, and the results were identical to the *in vivo* changes. In the *in vitro* tests, if the ESR was increased, the increase was proportional to the quantity of gelatin added to the blood.

TABLE II.

In vivo changes in ESR in dogs transfused with gelatin, and in vitro changes in mixtures of whole blood and gelatin and dextran.

IN VIVO:		WHOLE BLOOD AND GELATIN:				Per cent of dextran 50
Before.	After.	50	40	30	20	
0	34	64	...	9	4.5	66
0	40	54	47	41	5	76
0.5	61	56	32	19	16	57
0	0	0	0	0	0	0
0	0.5	1	1	1	1	0
38	...	83	74	72	50	83
21	...	72	57	45	30	...
7	...	73	63	43	30	...
0	...	1.5	1	1	0.5	...

DISCUSSION.

Barcroft (1926) calculated that contraction of the spleen during hæmorrhage can add to the stream 10 per cent of the natural circulating volume of blood. It has already been mentioned that from changes in spleen volume during hæmorrhage Wiggers (*loc. cit.*) calculated that 100 c.c. to 200 c.c. blood is added to the stream. We had measured the blood volume of two dogs before and after excision of spleen. In one dog the blood volume was reduced from 815 c.c. to 723 c.c., and in the other from 859 c.c. to 770 c.c., an average reduction of 6.8 c.c./kg. This figure is close to that of Overby (1946) who found an average reduction of 5.8 c.c./kg. due to splenectomy. In earlier experiments when spleen was not removed, after withdrawal of half of the blood and infusion of an expander, in 45 dogs out of 50, the total cell volume was 7 ± 6.7 per cent higher than the expected quantity, which was equivalent to 80 c.c. of blood. The quantity is very nearly the same as the difference in the blood volume before and after splenectomy. It is possible that in our experiments, due to a very brief duration of oligæmia the contribution from the spleen was not always maximal.

It is considered that the use of splenectomized dogs provides a rigorous and more accurate test for the comparative evaluation of plasma expanders. It removes a major inaccuracy in the estimation of blood volume by the dye method, and eliminates the passage of a variable quantity of blood from the spleen to the circulating volume during and immediately after hæmorrhage.

The effect on the ESR of the blood of dog and man is not alike. The red cells of man are invariably clumped by macro-molecular solutions, such as dextran and gelatin (Kalra *et al.*, *loc. cit.*), but in the case of certain dogs the cells remain dispersed.

SUMMARY.

1. MG-7 was tested on splenectomized dogs. All animals survived and the blood pressure was maintained at normal level in 6 dogs out of 12; in the rest it was 68 ± 4.4 per cent of the original level at the end of 4 hours.

2. The addition of balanced salt solution to gelatin helped in restoring the blood pressure quickly to normal and maintaining it at that level for one hour.
3. In 60 per cent of the dogs the red cells remained dispersed on *in vivo* or *in vitro* contact with gelatin and the ESR was not altered.
4. The use of splenectomized dogs to test plasma expanders is recommended.

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FURTHER STUDIES WITH NADISAN: EFFECT ON BLOOD CHOLESTEROL BLOOD, GLUTATHIONE AND SUGAR TOLERANCE IN ALBINO RATS.

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THE hypothesis that sulfonamides exert blood sugar-reducing effect by the chemical paralysis of alpha cells of islands of Langerhans has been supported by Loubatieres *et al.* (1955) and Von Holt *et al.* (1955). Selective destruction or paralysis of alpha cells by cobalt chloride has been shown by Caren and Carbo (1956) to cause increase in blood cholesterol, where the phase of hypercholesteremia always corresponds with the stage of degranulation of alpha cells.

In view of the above findings, it is necessary to consider the desirability of instituting prolonged sulphonamide therapy as advocated by Mohnike and Stotter (1956) in elderly diabetic patients who are usually prone to hypercholesteremia and atherosclerosis. Heightened peak value of sugar tolerance curve as a result of sulphadiazine and sulphanilamide administration in pancreatectomized dogs was observed by Mendez-Alvez (1951). Therefore, it appears interesting to determine how the carbohydrate tolerance is affected as a result of prolonged sulphonamide therapy.

In continuation of our previous work (Mukherjee, De and Mukherji, 1957, 1957a, 1958) the following experiments have been undertaken to study the effect of short and prolonged administration of nadisan on blood cholesterol, blood glutathione level and sugar tolerance in albino rats.

METHODS AND MATERIALS.

Albino rats of the Central Drug Research Institute breed, weighing approximately from 150 g. to 200 g., were selected for our experiment. The animals were

kept to stock diet *ad lib.* for a period of 7 days. The animals were then divided into 3 groups as follows:—

GROUP 1.—Twenty-four animals served as normal control. Blood was obtained from 8 animals of this group by heart puncture for estimation of cholesterol, glutathione and sugar; and glucose-tolerance test was performed on the remaining 16 rats.

GROUP 2.—Eight animals were fed nadisan 0.25 g./kg. each and glucose-tolerance test was performed on all the animals 2 hours after nadisan feeding.

GROUP 3.—Fifty-eight animals were fed 0.5 g./kg. of nadisan each and 2 hours after nadisan feeding, blood for cholesterol, glutathione and sugar estimation was collected from one batch of 8 animals by heart puncture and glucose-tolerance test was done on another 16 rats. The rest of the 34 animals were divided into 2 batches of 10 and 24 animals and nadisan feeding was continued for 14 days to the first and for 28 days to the second batch. Two days after the last nadisan feeding, glucose-tolerance test was performed on all the 10 animals of the first batch and 16 animals of the second batch. Blood was collected by heart puncture from remaining 8 animals of the second batch for cholesterol, glutathione and sugar estimations.

All the animals were kept without food for 16 hours prior to all tests but water was allowed *ad lib.* Glucose-tolerance test was performed by the method of Cole and Harned (1938). After collecting the blood for fasting sugar, glucose 3.5 g./kg. in 10 per cent solution was administered by intraperitoneal route. Subsequent blood samples for sugar estimation were collected at intervals of $\frac{1}{2}$, 1, 2, 3 and 5 hours after glucose. Blood was usually collected from tail vein unless otherwise mentioned. Blood sugar was estimated by Folin and Wu micro's (1920) method. Blood cholesterol was estimated by the method of Bloor (1916) and blood glutathione was estimated by the method of Woodward and Fry (1932). Urine passed during the test was collected and examined by Benedict's method. Nadisan was force fed suspended in water.

RESULTS.

In Table I, cholesterol and glutathione values of blood of both normal control and nadisan-fed animals have been shown. Average cholesterol and glutathione level in blood of normal control rats are 100 ± 0.8 mg. per cent and 38 ± 1.6 mg. per cent, respectively, and the corresponding average values in rats 2 hours after single (0.5 g./kg.) dose of nadisan are 103 ± 1.8 mg. per cent and 41 ± 2.3 mg. per cent, respectively. So, it becomes evident that cholesterol and glutathione level of blood in rats 2 hours after nadisan feeding do not materially differ from those of normal controls. Average cholesterol and glutathione values in rats after 28 days feeding of nadisan (0.5 g./kg./day) are 99 ± 5.2 mg. per cent and 39 ± 1.09 mg. per cent, respectively. These values also do not show any marked difference from those of normal controls.

The results of glucose-tolerance test in normal control animals show that maximum rise of blood sugar occurs $\frac{1}{2}$ hour after glucose administration followed by a sharp fall of blood sugar during the next $\frac{1}{2}$ hour (1 hour after glucose).

During the next two hours (2nd and 3rd hour after glucose), there was practically no change in the blood sugar level. But the blood sugar reaches the pre-glucose fasting level by the end of 5th hour after glucose. Glycosuria was present in 6 animals (Table II).

TABLE I.

Effect of Nadisan on blood glutathione and blood cholesterol in albino rats.

Rat number.	BLOOD CHOLESTEROL MG./100 C.C.:			BLOOD GLUTATHIONE MG./100 C.C.:		
	Control.	0.5 g./kg. Nadisan (single).	0.5 g./kg. Nadisan 28 days.	Control.	0.5 g./kg. Nadisan (single).	0.5 g./kg. Nadisan 28 days.
1	100	110	110	46	43	46
2	100	110	100	40	34	40
3	100	100	105	40	43	38
4	105	100	90	34	43	40
5	100	100	80	34	46	40
6	100	100	80	34	42	37
7	100	100	110	34	41	37
8	...	...	120	40	39	37
Mean $\pm$ S.E.	100 $\pm$ 0.8	103 $\pm$ 1.8	99 $\pm$ 5.2	38 $\pm$ 1.6	41 $\pm$ 2.3	39 $\pm$ 1.09

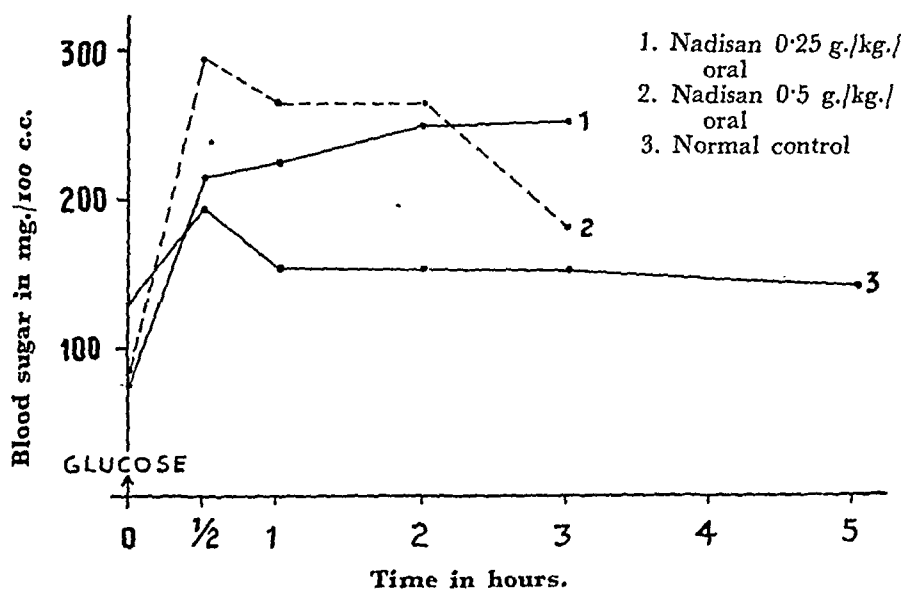
TABLE II.

Mean blood sugar values during glucose tolerance test in normal and Nadisan fed rats.

Number of animals.	Fasting B.S. (Mean $\pm$ S.E.) in mg./100c.c.	Dose of Nadisan fed.	BLOOD SUGAR (MEANS $\pm$ S.E.) IN MG. PER 100 C.G.:						Glycosuria present.
			Two hours after nadisan feeding.	After administration of glucose.					
				$\frac{1}{2}$ hour.	1 hour.	2 hours.	3 hours.	5 hours.	
16 (normal) control	136 $\pm$ 1.25	...	...	192 $\pm$ 3.6	153 $\pm$ 6.5	153 $\pm$ 6.4	153 $\pm$ 7.3	141 $\pm$ 6.3	In 6 out of 16.
8	136 $\pm$ 1.25	0.25g./kg. single dose	74 $\pm$ 4.4	214 $\pm$ 19.4	223 $\pm$ 36.9	250 $\pm$ 39.6	252 $\pm$ 42.8	...	In all.
16	136 $\pm$ 1.25	0.5 g./kg. single dose	76 $\pm$ 3.06	296 $\pm$ 14.1	266 $\pm$ 17.6	276 $\pm$ 27.7	181 $\pm$ 24.9	...	In all.
		2 days after discontinuation of Nadisan feeding							
10	136 $\pm$ 1.25	0.5 g./kg./day for 14 days	146 $\pm$ 2.3	255 $\pm$ 20.0	177 $\pm$ 14.9	158 $\pm$ 6.5	156 $\pm$ 8.2	148 $\pm$ 6.5	In all.
16	136 $\pm$ 1.25	0.5 g./kg./day for 28 days	121 $\pm$ 1.57	313 $\pm$ 17.2	248 $\pm$ 25.9	241 $\pm$ 16.2	206 $\pm$ 18.7	156 $\pm$ 12.8	In all.

In animals fed single doses of 0.25 g./kg. and 0.5 g./kg. of nadisan, the fasting preglucose blood sugar level, 2 hours after nadisan feeding, showed lower values than those of *normal control* animals. In animals which were fed nadisan 0.25 g./kg. single dose, blood sugar after glucose administration continued to rise up to 3 hour. But the animals which were fed nadisan 0.5 g./kg. in a single dose, blood sugar reached maximum height $\frac{1}{2}$ hour after glucose, though the blood sugar in these animals $\frac{1}{2}$ hour after glucose was higher than the corresponding values in animals fed 0.25 g./kg. nadisan, yet there was a definite fall of blood sugar up to 3 hour of observation. Observations in the above two experiments could not be continued beyond 3 hours. Glycosuria was present in all the animals (Graph 1).

GRAPH 1.



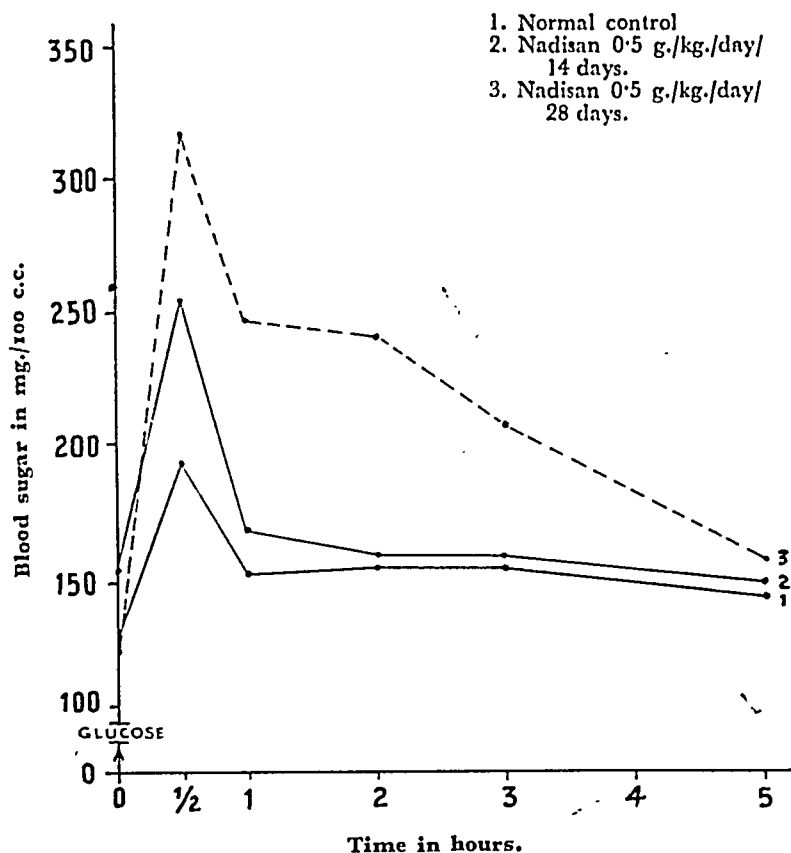
2 hours after Nadisan

The results of animals which were fed nadisan (0.5 g./kg./day) continually for 14 and 28 days show that pre-glucose fasting blood sugar levels, 2 days after last nadisan feeding, are more or less the same as in normal control. Maximum rise of blood sugar in both the batches occurs $\frac{1}{2}$ hour after glucose administration but the height of blood sugar level at this period in 28 days fed batch of animal reaches a higher level than that of 14 days fed batch of animals. In 14 days fed animals blood sugar reaches the pre-glucose fasting level by the end of 5th hour, whereas in 28 days fed animals, though the blood sugar was coming down, fail to reach the pre-glucose fasting level by the end of 5th hour. Glycosuria was present in all the animals (Table II and Graph 2).

DISCUSSION.

The data show that nadisan feeding, either in a single dose, or in repeated doses for a long time, do not cause any alteration in the cholesterol level of blood in albino rats. This finding is not in agreement with that of Hartel and Antila (1956) who observed definite increase of blood lipoid and cholesterol in animals after

GRAPH 2.



nadisan feeding Kinsell *et al.* (1956) suggested that the primary action of BZ-55 is on fat metabolism which he linked with the antidiabetic nature of BZ-55 but our results do not tend to support this view.

The protective power of an agent against the diabetogenic action of alloxan depends, as previously stated (Mukherjee *et al.*, 1955) upon its ability to elevate and maintain the glutathione level of blood. Our present results show that oral administration of nadisan has little effect on the blood glutathione level and this may be one of the reasons for failure of nadisan to prevent the diabetogenic action of alloxan as previously observed (Mukherjee *et al.*, 1958).

The results of glucose-tolerance tests show that in normal control animals the maximum rise of blood sugar occurs $\frac{1}{2}$ hour after glucose and the peak value never exceeds 200 mg. per cent. In nadisan-fed animals, also, the rise of blood sugar is maximum $\frac{1}{2}$ hour after glucose administration, except in animals fed 0.25 g./kg., but the peak values in all cases exceed those of normal control. Mendez-Alvez (*loc. cit.*) also observed similar heightened peak of sugar tolerance curve following daily administration of non-hypoglycemic sulpha drugs to depancreatized dogs for 3 days.

Another feature of tolerance curve in nadisan-fed animals is that the peak value goes on increasing with increased dose as well as with increased duration of nadisan feeding. The results of two single dose of nadisan used in our experiment show that higher dose produced less degree of defect in the sugar tolerance curve as the pre-glucose fasting blood sugar value was nearly reached although the peak value after glucose was heightened, whereas with lower dose there was progressive rise of blood sugar. When the same dose of nadisan feeding is continued, animals fed for shorter period (14 days) show better tolerance of sugar compared to the animals fed for longer period.

The reason or reasons for these abnormalities are not clear. If sulfonamides produces hypoglycemia by stimulating insulin secretion by its action on beta cells as explained by Loubatieres (1944, 1944a, 1946) then enhanced and prolonged hyperglycemia due to load of glucose in nadisan fed animals may probably result from temporary non-availability of insulin due to exhaustion of insular-apparatus. Unbalanced alpha cell activity in such conditions probably may also indirectly contribute to the derangement of sugar metabolism. It will be too early at the moment to conclude as to whether unrestricted feeding of nadisan for a very long time will cause sufficient degree of exhaustion of beta cells leading to permanent derangement of the function of insular apparatus.

As the dose used in rats is considerably higher than the usual clinical dose and since the metabolism in rats is different from that of human being any such adverse effect of continued administration of nadisan on sugar tolerance in diabetic individuals cannot be correctly predicated.

SUMMARY.

1. Effect of nadisan feeding in different doses and period on blood cholesterol, blood glutathione (reduced) and sugar tolerance in albino rats has been studied. It has been observed that nadisan feeding (0.5 g./kg.) either in a single dose or in repeated doses does not alter the cholesterol or glutathione (reducing) level of blood in albino rats.

2. Sugar tolerance curves in nadisan fed animals are defective in the sense that peak value in all the nadisan-fed animals is much higher than that of normal and the return of the blood sugar to the pre-glucose level is much delayed.

Thanks are due to Messrs. M. A. Hai and H. M. Chakravarti for their technical assistance and to Mr. S. Banerji for drawing the graphs.

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OBSERVATIONS ON THE PHARMACOLOGICAL ACTIVITY OF DIFFERENT SALTS OF ADRENALINE, MORPHINE AND STRYCHNINE.

BY

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FOR therapeutic uses, very often more than one salt of an alkaloid is available in the market and it is sometimes difficult to decide, which one should be preferred over others. For example, adrenaline, strychnine and morphine, are available as adrenaline hydrochloride and tartrate, strychnine hydrochloride and sulphate, morphine sulphate, hydrochloride and tartrate. These salts are usually prepared, bearing in mind their solubility, local reaction and also stability of solution. No systematic study seems to have been made about their reactions in the body and whether they can produce any quantitative difference in pharmacological responses on animal tissues. As molecular weight of different salts of an alkaloid also differs appreciably, it might be interesting to investigate as to whether this would have any bearing with the quantitative responses of the drug.

With these points in view, it was considered necessary to carry out a series of investigations with the different salts of adrenaline, strychnine and morphine in order to evaluate their pharmacological actions. The observations are contained in the present paper.

EXPERIMENTAL.

Material.—For the present investigation, bases of adrenaline (natural and synthetic), morphine and strychnine, conforming to B. P. tests of identity and purity, were obtained. In case of adrenaline, the optic rotation was also determined by Zeiss-Winkle polarimeter. Hydrochloride, citrate and tartrate salts of adrenaline, hydrochloride, sulphate and tartrate salts of strychnine and hydrochloride, sulphate, meconate and tartrate salts of morphine were prepared volumetrically with calculated amounts of acids, taking into consideration the mono- and dibasicity of these acids. The quantity of powder, used for the preparation of solutions, was

the same in each case and was measured on a micro-analytical balance. As far as possible, freshly prepared solutions, stabilized with suitable stabilizers, were used for the assay.

The molecular weights of various salts, used in the present study, are given in Table I:—

TABLE I.

Showing molecular weight of salts of adrenaline, morphine and strychnine.

Salt.	MOLECULAR WEIGHT:		
	Adrenaline.	Morphine.	Strychnine.
Hydrochloride	219·7	321·8	370·9
Sulphate	...	668·8	...
Tartrate	333·3	720·7	484·5
Citrate	375·3	477·4	526·5
Meconate	...	770·6	...

Methods.—The assay of the 3 alkaloids was carried out by the determination of pressor effect in case of adrenaline, analgesic and respiratory depressant actions in case of morphine and convulsant action in case of strychnine salts.

Pressor potency of adrenaline salts was studied by Elliott's method in spinal cats, previously anæsthetized with a minimum quantity of ether. About two hours after decerebration, when the animals were stabilized, they were injected with a series of maximal doses of adrenaline solution with a view to saturate the animal with the drug before the assay work was actually started. Under these conditions the animal variation could be reduced to a minimum and reasonably stable carotid pressure of 30 to 50 mm. Hg. could be established. The assay was then carried out at a sub-maximal level of response with doses varying between 10 μ g. and 25 μ g. in different animals.

Analgesic potency of morphine salts was determined by Davies, Raventos and Walpope (1946) method based on the prolongation of withdrawal of rat tail, kept on heated nichrome wire of the analgesiometer. Investigations were carried out on rats, which had a constant reaction time from the moment of closure of the key to the withdrawal of the tail. They were then divided into 3 groups, of 10 rats each and injected with graded doses of 1 to 3 mg./kg. of morphine salts. The threshold for reflex withdrawal of the tail was determined every $\frac{1}{2}$ hour till the reaction time returned to the original level. The total increase in reflex withdrawal time was calculated in each rat and average increase after a single dose of morphine salt was calculated. 'Cross-over tests' were performed after a week.

Potency of morphine salts was also determined on the basis of their ability to depress respiration in anæsthetized, tracheotomized dogs.

Convulsive potency of strychnine was determined by administration of graded doses of different salts on batches of 10 frogs and percentage response observed for one hour in each case. Intensity and duration of hyper-reflexia were compared in each group, and scores of 1 to 4 *plus* given.

RESULTS.

Our observations in respect of actions of different salts of adrenaline, morphine and strychnine are detailed below.

Adrenaline salts.—The effect of hydrochloride, citrate and tartrate salts of adrenaline, on the pressor response of spinal cats is given in Table II:—

TABLE II.

Showing comparative pressor effect of adrenaline salts, the hydrochloride being taken as a standard with an arbitrary value of hundred per cent.

Number of observations.	PER CENT POTENCY:			Power of discrimination of the animal, $\mu\text{g./kg.}$
	Adrenaline hydrochloride.	Adrenaline citrate.	Adrenaline tartrate.	
1.	100.0	105.2	113.6	0.2
2.	100.0	117.7	117.6	0.5
3.	100.0	105.2	117.6	0.6
4.	100.0	113.6	129.8	0.5
5.	100.0	107.5	132.8	0.5
6.	100.0	100.0	136.9	0.3
7.	100.0	111.1	128.5	0.3
8.	100.0	111.1	121.9	0.3
9.	100.0	113.6	117.6	0.6
10.	100.0	117.6	132.8	0.3
Average ...	100.0	109.6 ± 5.1	124.9 ± 8.0	0.4 ± 0.14

It will be seen from Table II that in the present investigations with the three salts of adrenaline, the tartrate has the maximum, the citrate intermediate and the hydrochloride minimum (124.9 per cent, 109.6 per cent and 100 per cent, respectively) pressor effect. An examination of the last column of the table indicates that the experimental animals were sufficiently sensitive and able to discriminate between 0.2 and 0.6 $\mu\text{g./kg.}$ doses.

Morphine salts.—The analgesic and respiratory depressant actions of different salts of morphine are contained in Tables III and IV:—

TABLE III.

Showing analgesic potency of morphine salts based on their ability to increase reaction time in rats.

Number of observations.	Doses, mg./kg.	AVERAGE TOTAL INCREASE IN REACTION TIME, SEC.		
		Morphine hydrochloride.	Morphine sulphate.	Morphine tartrate.
10	1.0	27.0	41.0	45.2
10	2.0	38.6	44.9	55.6
10	4.0	44.6	49.7	62.5
10	8.0	57.4	63.5	71.2

TABLE IV.

Showing comparative respiratory depression produced by different salts of morphine in chloralosed dogs.

Dosage, mg./kg.	AVERAGE PERCENTAGE DEPRESSION OF RESPIRATORY RATE:			
	Morphine hydrochloride.	Morphine sulphate.	Morphine meconate.	Morphine tartrate.
0.5	22.4	29.9	26.6	40.4
1.0	26.2	36.6	39.5	52.6
2.0	32.5	51.2	48.0	58.2

It is evident from Tables III and IV that morphine tartrate produced maximum analgesia and respiratory depression, morphine sulphate was intermediate in analgesic potency and morphine hydrochloride showed minimum effect. Respiratory depression after meconate and sulphate salts was approximately of the same order.

In general, it was observed that the development of tolerance was also maximum with the tartrate salt. The duration of analgesic action after a single subcutaneous dose, lasted for $3\frac{1}{2}$ to 5 hours after tartrate, 2 to 4 hours after sulphate and 2 to 3 hours after use of the hydrochloride salt.

In another series of experiments in which the comparative effect of morphine salts in abolishing the reflex increase in respiratory rate after stimulation of the central cut-end of sciatic nerve was studied, it was observed that morphine tartrate and meconate showed higher activity than the hydrochloride and sulphate salts.

Strychnine salts.—The relative convulsive potency of different salts of strychnine is shown in Table V:—

TABLE V.

Showing comparative convulsant action of different salts of strychnine in frogs.

	Dosage, mg./kg.	Strychnine hydrochloride.	Strychnine citrate.	Strychnine tartrate.
Percentage convulsed ...	0.12 0.25	47.14 54.50	60.0 80.0	58.8 70.0
Intensity of hyper-reflexia ...	0.12 0.25	++ +++	++ +++±	++ ++±
Onset of convulsion (minutes) ...	...	15—20	20—30	25—40
Duration of hyper-reflexia (hours)	0.12 0.25	2.4 3.5	1.3 2.4	3.4 4.5

It will be seen from Table V that strychnine citrate and tartrate produced almost similar percentage of response and intensity of hyper-reflexia in frogs. The onset of convulsion was, however, more prompt with the hydrochloride and the duration of action greater after the tartrate salt. Of the three solutions under investigations, the aqueous solution of strychnine hydrochloride appeared to be the most stable.

DISCUSSION.

In the present study, comparative effect of different salts of adrenaline, morphine and strychnine has been investigated with a view to find out whether there is any inter-relationship between their molecular weight and quantitative pharmacological responses.

From the work with adrenaline salts, it has been observed that adrenaline tartrate has the maximum pressor effect in spinal cats, next in order being citrate and finally hydrochloride. A reference to Table I shows that the hydrochloride salt has the lowest molecular weight than tartrate and finally citrate salts. So far as their pressor potency is concerned, this is the lowest for the hydrochloride and maximum for the tartrate salt, the citrate being intermediate in this respect.

This may be due to the fact that the citrate salt, being readily metabolized in the body, may be less stable than the tartrate, which might subscribe to its somewhat lower pressor potency. Further, the combination of laevo adrenaline with the optically active dextrorotatory tartaric acid might also enhance its activity in spite of the ultimate dissociation of respective ions in the body.

On analysis of the salts of morphine, it has been observed that the analgesic potency is in proportion to their molecular weight. As attempts have been made to correlate basicity, ionization constant and pH in case of pressure amines and sedatives (Percy May, 1921; Burger, 1951; and David, 1955), it is likely that di-basicity of morphine tartrate, meconate and sulphate may also be responsible for their more intense and prolonged action than the hydrochloride. Similarly, weaker depressant action of morphine meconate in spite of its higher molecular weight than tartrate might be explained on the basis of its limited solubility and rate of dissociation as compared to the tartrate salt which produced marked effect. It may further be observed that of the various salts of morphine studied in this series, the hydrochloride is mono basic and it has also shown the minimum effect.

In the strychnine series, where the drugs were injected into the dorsal lymph sac of frogs, with lower doses (0.12 mg./kg.), the convulsant effect was found to be closely similar for the 3 salts but with 0.25 mg./kg. doses, the citrate and tartrate salts showed higher activity. The hydrochloride salt, however, produced a prompter response probably due to its faster rate of ionization.

From the foregoing, it would be observed that molecular weight seems to have some relationship with pharmacological actions of a salt but at our present stage of knowledge, no broad generalization is possible. Drug action being a complicated mechanism, other physical properties, such as solubility, permeability, basicity, ionization constant, optical rotation, etc. might also play important roles in their effectiveness.

SUMMARY AND CONCLUSIONS.

1. In the present paper, investigations have been carried out with different salts of adrenaline, morphine and strychnine with a view to ascertain as to whether changes in the molecular weight could produce quantitative difference in responses on animal experimentation.

2. In case of adrenaline, it has been observed that the tartrate salt, in equimolecular doses, produced greater pressor effect in spinal cats than the hydrochloride and citrate salts.

3. In case of morphine also, the tartrate salt has been found to produce maximum analgesic and respiratory depressant action in rats and dogs as compared to the hydrochloride, sulphate and the meconate salts.

4. In case of strychnine, the citrate and tartrate salts have been found to possess maximum convulsant action in frogs as compared to the hydrochloride salt.

5. The role of molecular weight in this increased quantitative response, its limitations and the role of other factors such as solubility, permeability, dissociation constant, basicity, etc. has been discussed.

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EFFECT OF TESTOSTERONE PROPIONATE ON THE ADRENAL CORTEX OF THYROXINE-TREATED RATS.

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A concurrent stimulation of the adrenal cortex and hypertrophy of the thyroid was noted by Roy, Roy and Karkun (1957) in rats treated with testosterone propionate. Thyroid hormone plays an important rôle in regulating the adreno-cortical activity (Selye, 1950; Williams, 1955) and it stimulates the adrenal cortex by augmenting the secretion of ACTH from the pituitary (Smith, 1930; Eartly and Leblond, 1954). The above observations, therefore, raised a question whether the adrenal stimulating action of testosterone requires the mediation of thyroid or not. Such a question becomes pertinent in view of the finding that while the androgen can stimulate the adrenals of experimental hypothyroid rats, the degree of stimulation is lessened when the hypothyroidism is of longer duration (Roy, Karkun and Sur, 1958).

Roy, Roy and Mukerji (1956) have shown that thyroxine blocks the thyroid stimulating action of the androgen. A study of the influence of testosterone propionate on the adrenal cortex of thyroxine-treated animals was, therefore, undertaken with a view to elucidate further the rôle of thyroid gland in the action of the testoid on the adrenal cortex.

EXPERIMENTAL PROCEDURE.

Thirty-two young male albino rats of the Institute colony, weighing 41.1 ± 4.1 g., were divided into four groups of 8 each. To one group thyroxine (15 μ g.,

dissolved in 0.4 c.c. of distilled water/rat daily) was administered orally for 13 days. Another group received intramuscular injections of testosterone propionate (6 mg. dissolved in 0.2 c.c. of sesame oil/rat on alternate days) over the same period of time. The third group had the combined treatment with thyroxine and testosterone propionate in the above dose schedule. The remaining group served as a control receiving only the diluents. All were maintained under uniform husbandry conditions.

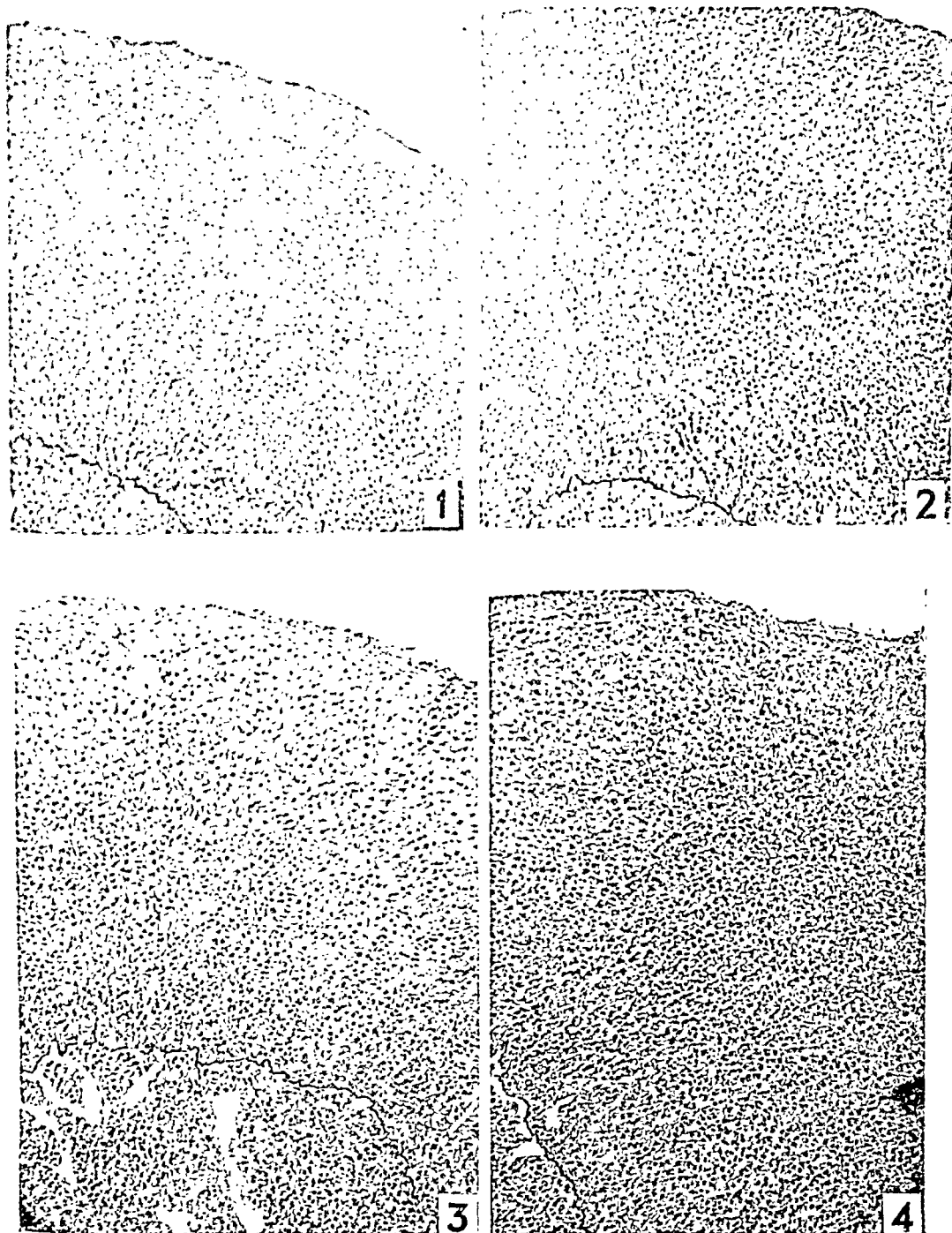
The animals were sacrificed on the day following the final hormone treatment. The adrenals were carefully dissected out, freed from adhering fat and weighed in a microbalance. The left adrenals were processed for the determination of ascorbic acid and the right ones for the total cholesterol contents. The estimations of ascorbic acid and total cholesterol contents were done according to the methods described by Karkun *et al.* (1954) and Roy *et al.* (1955), respectively. Six adrenals were allotted for each biochemical estimation. The rest of the glands were spared for histological examinations and were fixed in 10 per cent formal saline. The thyroid glands were also suitably fixed for histological studies. Serial paraffin sections of the glands were stained with Ehrlich's hematoxylin and counterstained with cosin.

RESULTS.

Table I shows the weight, ascorbic acid and total cholesterol contents of the adrenals of different groups of animals. It is seen that testosterone propionate increases the weight of the adrenal and the ascorbic acid and total cholesterol contents of the gland record a fall. Thyroxine treatment seems to bring about an increase in adrenal weight but the change fails to become statistically significant. The ascorbic acid and total cholesterol contents of the adrenals in this group are, however, significantly lowered from those of the control. The adrenals of the combined treated group show marked enlargement and they are significantly heavier than those of the animals treated with testosterone alone. The cholesterol contents, too, show a greater decline. The ascorbic-acid content, however, though significantly lower than that of the control, is virtually at the same level as that observed in the testosterone-treated animals.

Histological picture of the adrenals of the control animals presents a normal appearance of the cortical parenchyma (Plate X, fig. 1). The sinusoids are noticeable in between the columns of cortical cells. Medullary cells present a normal appearance.

The adrenals in the testosterone treated group (Plate X, fig. 3) show considerable enlargement of the cortex. Cells of the zona glomerulosa show hypertrophy. Considerable hypertrophy and hyperplasia of the cells are observed in the zona fasciculata and increased mitosis is noticeable. Some of the cells of zona reticularis and a few cells of the zona fasciculata show fatty metaplasia. Fat vacuoles are seen in the cytoplasm of the cells and the nuclei are pushed to one side giving the cells a typical signet-ring appearance. But this change is not consistently noted in all the adrenals. Vascularity of the gland is increased. Medullary cells present a normal picture.



(All figures are photomicrographs, $\times 120$)

- FIG. 1. A section through the adrenal of a normal rat.
- FIG. 2. A section through the adrenal of thyroxine-treated rat. Note the hypertrophy of the cortex. Compare with Fig. 1.
- FIG. 3. A section through the adrenal of testosterone propionate-treated rat. Note the hypertrophy of the cortex and fatty metaplasia of some of the cells of the inner cortical zones. Compare with Figs. 1 and 2.
- FIG. 4. A section through the adrenal of thyroxine *plus* testosterone propionate-treated rat. Note greater hypertrophy of the cortex and greater extent of fatty metaplasia of the cortical cells in comparison with those noted in testosterone treated group. Compare with Figs. 1

TABLE I.

The weight, ascorbic acid and cholesterol content of the adrenals of control and experimental rats.

Group and treatment.	Mean body-weight:		Mean adrenal weight (mg.) with S.E.	Mean ascorbic content (mg./100 g. of adrenal tissue) with S.E.	Mean cholesterol content (mg./g. of adrenal tissue) with S.E.
	Initial, g.	Final, g.			
A. Control	41.1	58.5	15.6 $\pm$ 0.97	580 $\pm$ 6.5	19.03 $\pm$ 1.34
B. Testosterone propionate	41.0	57.1	22.4 $\pm$ 0.61	296.6 $\pm$ 10.5	15.07 $\pm$ 0.93
C. Thyroxine	41.5	50.6	17.9 $\pm$ 0.64	509.2 $\pm$ 16.4	15.0 $\pm$ 0.92
D. Testosterone propionate plus thyroxine	41.8	54.4	24.7 $\pm$ 0.77	300 $\pm$ 0	12.27 $\pm$ 1.18

Analysis.—

Adrenal weight:

A vs. B	t=5.938	P<.001	(Significant)
A vs. C	t=1.982	P<.1>.05	(Insignificant)
A vs. D	t=7.280	P<.001	(Significant)
B vs. D	t=2.363	P<.05>.02	(Significant)
C vs. D	t=6.868	P<.001	(Significant)

Adrenal ascorbic acid:

A vs. B	t=23.452	P<.001	(Significant)
A vs. C	t=4.036	P<.01>.001	(Significant)
A vs. D	t=43.076	P<.001	(Significant)
B vs. D	t=0.323	P>.7	(Insignificant)
C vs. D	t=12.834	P<.001	(Significant)

Adrenal cholesterol:

A vs. B	t=2.459	P<.05>.02	(Significant)
A vs. C	t=2.318	P<.05>.02	(Significant)
A vs. D	t=3.797	P<.01>.001	(Significant)
B vs. D	t=1.879	P<.1>.05	(Insignificant)
C vs. D	t=1.844	P<.1>.05	(Insignificant)

The adrenals in the thyroxine-treated animals show some enlargement of the cortex due to hypertrophy and hyperplasia of the cells of zona fasciculata (Plate X, fig. 2).

Histological picture of the adrenal in the combined treated group (Plate XI, fig. 4) is more or less same as that observed in the adrenals of testosterone-treated animals. But the fatty metaplasia of the cortical cells are found consistently and seem to be more pronounced in this group. This change occurs in some of the cells of zona reticularis and zona fasciculata. Increased vascularity of the gland is noticeable.

Microscopical examination of the thyroid reveals that testosterone causes hypertrophy and hyperplasia of the acinar cells. Thyroxine treatment produces atrophy of the acinar cells both in normal and testosterone-treated animals. It thus becomes quite evident that the former hormone has blocked the thyroid-stimulating action of the latter.

DISCUSSION.

Data presented in this study show that testosterone stimulates the adrenal cortex of the rat. It produces hypertrophy of the adrenal and lowers the ascorbic acid and total cholesterol contents of the gland. These findings corroborate the previous observations by Roy, Roy and Karkun (*loc. cit.*). But here, the fatty metaplasia is not observed consistently and is of lesser degree in comparison to that observed in the previous study. It may be due to the fact that the duration of treatment with the testoid in the present experiment was less than that of the previous one.

Changes in the weight of the adrenal along with those in the chemical constituents indicate that thyroxine also stimulates the adrenal cortex. These observations are in agreement with those by previous investigators (Selye, *loc. cit.*, Kar *et al.*, 1955).

The weight of the adrenals in the combined treated group is greater not only than that of the controls but also than that of either thyroxine or testosterone-treated animals. In fact, the enlargement of the adrenals reflects the additive effect of the influences of the two hormones. Degree of depletion of the cholesterol content, too, in this combined treated group, appears to point out the same additive effect. The change in the vitamin content is, however, the same as noted in the animals treated with testosterone alone. The reasons for the discrepancy in the behaviour of the two chemical constituents is not clear. Of course, it has been seen that the changes in these two constituents of the adrenal do not always run parallel. (Tepperman *et al.*, 1947; Pinchot *et al.*, 1949).

It is thus seen that testosterone exerts its stimulating influence on the adrenal cortex in spite of the fact that the thyroid-stimulating action of the sex hormone is blocked by thyroxine. Moreover, it appears that the additive effect of the two hormones is obtained in the combined treated group. The changes in the adrenal weight and the cholesterol content of the gland at least support such a viewpoint. It seems, therefore, quite reasonable to conclude that this adrenal stimulating action of testosterone propionate does not mediate through the thyroid gland.

The fatty metaplasia of the adrenal cortical cells as noted in the testosterone-treated animals requires some comment. This fatty change is presumably due to peripheral interactions between testosterone and the adrenocorticotrophic hormone. It fails to develop in the absence of the latter hormone but becomes, particularly, more pronounced in those cases where the animals are simultaneously treated with corticotrophic preparation in addition to testosterone (Selye, *loc. cit.*). Our present observations seem to be significant in this respect. Though there occurs inconsistently some amount of fatty metaplasia of the adrenal cortical cells in the rats treated with testosterone alone, this change becomes consistent and more prominent in the animals treated with thyroxine in addition. Probably, the increased amount of ACTH secreted by thyroxine (Eartly and Leblond, *loc. cit.*) accentuate the fatty metaplasia of the adrenocortical cells brought about by testosterone treatment.

SUMMARY.

Administration of testosterone propionate causes stimulation of the adrenal cortex of normal and thyroxine-treated rats producing hypertrophy and hyperplasia of the cells and causing depletion of the total cholesterol and ascorbic-acid contents of the gland. Fatty metaplasia of the cortical cells brought about by testosterone administration seems to be enhanced following simultaneous treatment with thyroxine. The significance of these findings in the elucidation of the mechanism of action of testosterone are discussed.

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DIETHYLCARBAMAZINE (HETRAZAN) IN TROPICAL EOSINOPHILIA.

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INTRODUCTION.

WEINGARTEN (1943) described his accidental discovery of arsenic as a specific therapeutic agent in the treatment of tropical eosinophilia. Since then, many other workers have confirmed the effectiveness of various organic arsenicals in this disease (Heilig and Visveswar, 1943; Misra *et al.*, 1953; Patel, 1945; and Vishwanathan, 1948). Clinical cure has been described in 50 per cent to 98 per cent of the patients after the use of arsenicals (Chaudhuri *et al.*, 1954; and Vishwanathan, *loc. cit.*). In the majority of patients, symptomatic relief is nothing short of dramatic. Most of the workers have preferred to use neo-arsephenamine or acetylarsen. Oral preparations, such as carbarsone, are considered to be less effective than the injected material but good comparative studies are lacking. Unfortunately, most of the therapeutic work in this disease was not accompanied by adequate blood examinations during or after the treatment.

Why arsenic should hold such a unique therapeutic effectiveness is an intriguing question. Arsenic might act upon a yet unidentified parasite which invades the human host or it may be effective as a cytoplasmic poison, depressing the hæmopoietic activity or selectively interfering with eosinophil formation (Reeder and Goodrich, 1952).

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Any other drug, which has parasitocidal or bone-marrow depressant activity might be effective in tropical eosinophilia. In the past, various other drugs had been experimented with. Bismuth and antimony were tried in vain (D'Abrera, 1946). Sulpha drugs and penicillin were used with uncertain effects (Joseph, 1946; and Chaudhuri *et al.*, *loc. cit.*). Somewhat better results have been reported with aureomycin (Chaudhuri, 1950; and Rao and Krishnan, 1952). However, this drug does not seem to be as effective as arsenic and is much more costly. ACTH and cortisone produce a prompt remission but a relapse occurs as soon as the drug is withdrawn (Chaudhuri, 1951; Chaudhuri *et al.*, *loc. cit.*; and Reeder and Goodrich, *loc. cit.*). We tried WIN 5047 (newer amebicidal drug), mepacrine, chloroquine, aminopterin (Ganatra and Lewis) and hetrazan in this condition. Results with aminopterin are referred to, subsequently in this article. All the drugs tried by us, except hetrazan, were found to be without any effect on the course of the disease. A preliminary report on the effectiveness of hetrazan in tropical eosinophilia was published by Ganatra and Lewis (1955). The results of subsequent trials of this drug, used in different doses, are reported here.

There are various genuine difficulties in establishing a well-controlled clinical trial in this condition. Even though the clinical features of this syndrome are fairly distinct, there are many patients with high eosinophilia and bizzare symptoms. There is no linear correlation between the severity of symptoms and the degree of eosinophilia. A basic diagnostic limit of eosinophilia is not set. Evaluation of the effectiveness of a drug cannot be based upon the relief of clinical symptoms only, as they are liable to great subjective variations. Differential eosinophil counting is subject to a great deal of technical error and can lead to a false impression during the follow-up of the patient. The natural course of the disease is not well known, however, it is certain that the disease is characterized by frequent spontaneous remissions. An attempt has been made to obviate these difficulties at various stages, in the present trial.

METHOD.

Results of treatment in 94 consecutive patients of tropical eosinophilia, treated with different dosages of hetrazan are recorded. These results are compared with results obtained in two control groups. The first control group consisted of patients who were kept without any specific treatment, the second control group comprised of patients who were treated with carbarsone. All the patients under observation were outdoor patients, and all the therapeutic trials were conducted on an ambulatory regime.

Selection of patients.—The selection of patient for observation was based on the presenting complaints and the initial total eosinophil count. Each patient was required to have predominant respiratory symptoms. Usually, these symptoms were cough and difficulty in breathing. Cough was dry, hacking, paroxysmal, nocturnal and accompanied by scanty expectoration. Difficulty in breathing was of various types. Some patients had just a choking sensation after a bout of cough, some felt actually dyspnoeic after such a paroxysm and a few of the patients had wheezing expiratory dyspnoea typical of bronchial asthma.

Various constitutional symptoms, such as malaise, lassitude, anorexia and loss of weight were also noted.

Clinical symptoms were graded according to their severity. Patients with cough, repeated asthmatic attacks and marked constitutional symptoms were classed as 3 *plus* severity, patients having cough and occasional attacks of difficulty in breathing were put as 2 *plus* severity, and all the patients with cough only, were grouped in a class marked 1 *plus* severity.

The total white blood cell and the total eosinophil count was done in each patient before and repeatedly after the course of treatment. The total eosinophil counts were done by the modified Pilot's method, as described by McFarlane and Cecil (1951). No patient was taken in the present series unless his total eosinophil count was above 5,000 cells per c.mm. The fact, that patients having counts lower than this may suffer from tropical eosinophilia is not denied, but this limit was fixed with two views; eosinophilia lower than this limit may be due to various other causes, and a fall in the total eosinophil count cannot be well appreciated if the initial count is below 5,000 cells per c.mm.

It was not feasible to take a skiagram in all the patients, but fluroscopy was performed repeatedly during and after the treatment. The findings on the physical examination of the patient and on fluoroscopy were useful mainly to exclude any other concurrent condition from which the patient might be suffering.

In 40 consecutive patients, routine examination of fresh stools was done. Hetrazan is known to be effective in certain helminthic infestations of the intestinal tract, and these examinations were undertaken to ascertain if the presence of any particular helminth was characteristic of tropical eosinophilia.

Allocation of patients in different treatment groups.—It was decided to have the following 8 groups of patients:—

(1) A group comprising of patients who were not given any specific treatment. It was desired to keep this group without a specific treatment for an indefinite period. They were given placebos during the period of observation. The period of observation was not constant in all the patients, but in all of them at least one blood examination was done after a period of 8 days.

(2) A group of patients treated with 0.26 g. of carbarsone twice daily for ten days.

(3) A group of patients treated with 13 mg./kg. of hetrazan daily for four days.

(4) A group of patients treated with 13 mg./kg. of hetrazan daily for 8 days.

(5) A group of patients receiving 6 mg./kg. of hetrazan daily for 4 days.

(6) A group of patients receiving 6 mg./kg. of hetrazan daily for 8 days.

(7) A group of patients who were given 3 mg./kg. of hetrazan daily for 8 days.

(8) A group of patients who were given 26 mg./kg. of hetrazan in 24 hours administered in three divided doses.

Each group was taken up consecutively. Only when the required quota of patients were allocated to one group, the subsequent group was embarked upon. Thus, essentially the distribution of patients in each group was at random. The first two groups were supposed to act as controls to all the other groups. It was desired to have about 30 patients in each of these two control groups, and to have about 20 patients in each of the subsequent groups. It will be seen that number of patients in each group was somewhat less because some of the patients had to be eliminated from the series due to a lack of sufficient follow up study.

Observations during the treatment period.—No drug was used for symptomatic relief during the course of treatment with the specific drug under trial. Usually, the patients were given a four days' quota of the specific drug with definite instructions about its administration. After this period, he was re-evaluated as regards symptoms. A blood examination was usually done at the end of the treatment period, but occasionally during treatment.

Follow-up.—Atleast a month's follow-up after the end of the treatment was aimed at, and a minimum of one blood examination was desired during each week of this month. This was not possible in many of the cases.

A period between the 8th and the 12th day after the initiation of the treatment, was designated as the 'critical period', and one blood examination during this period was always sought for. As this period was kept uniform in all the groups, a good comparative evaluation of the per cent fall in the total eosinophil count could be made.

During the follow-up period patients were given placebos.

Criteria for cure.—Improvement in a patient was judged on the basis of amelioration of his presenting symptoms and the degree of fall in his initial total eosinophil count. Emphasis was mainly on the latter factor. The treatment was considered successful, if the patient had a total eosinophil count below 2,500 within one month following the treatment. In case he could not be followed up for this period, if he had a fall of more than 50 per cent during the 'critical period' the treatment was also considered as successful. If a second blood examination within a fortnight after the critical period showed a rise in the eosinophil count, the treatment was judged to be a failure. In a patient in whom a significant fall in the total eosinophil count was not accompanied by symptomatic improvement, the results were called equivocal, but this situation was rare. A statistical comparison between the different groups was attempted on the basis of percentage fall in the eosinophil count during the critical period and also on the number of treatment failures as judged by the above criteria.

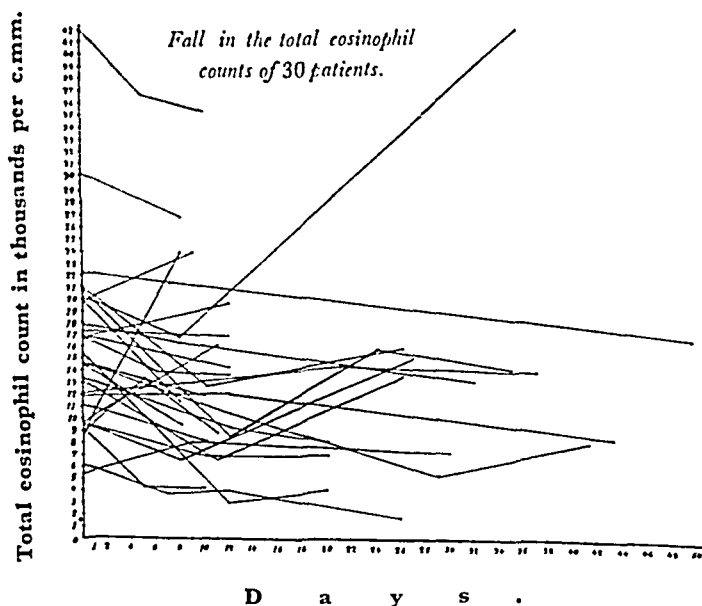
RESULTS.

One hundred and fifty-one patients were observed during the period between February 1955 and September 1955. Ninety-four of these patients were treated with hetrazan, 27 patients were treated with carbarsone and 30 patients were kept without any specific treatment.

I. *Group without any specific treatment.*—Thirty patients were kept without specific therapy for a minimum period of 8 days (Graph 1). Thirteen patients

were observed till second week, 5 patients till third week, 4 patients till fourth week and 8 patients were observed for more than one month. Maximum period of observation attained in one patient was 79 days. Each observation consisted of assessment of symptoms and blood examination.

GRAPH 1.



According to the criteria defined above, only in 3 patients the outcome was favourable. Even so, none of these patients had complete symptomatic relief, and only one patient attained eosinophil count lower than 2,500.

Graph 1 shows that most of the total eosinophil counts during the follow up period were persistently higher than 5,000. Mean fall in the total eosinophil count during the critical period (i.e. between 8th and 12th day) was only 11 per cent, with standard deviation of 46 and standard error of 9 (Table II). Fall of more than 50 per cent in the eosinophil count during this period was observed only in 3 patients, of which one showed a subsequent rise (Table III).

II. *Group treated with carbarsone.*—One tablet of 0.26 g. of carbarsone was administered twice daily for 10 days to 27 patients (Graph 2).

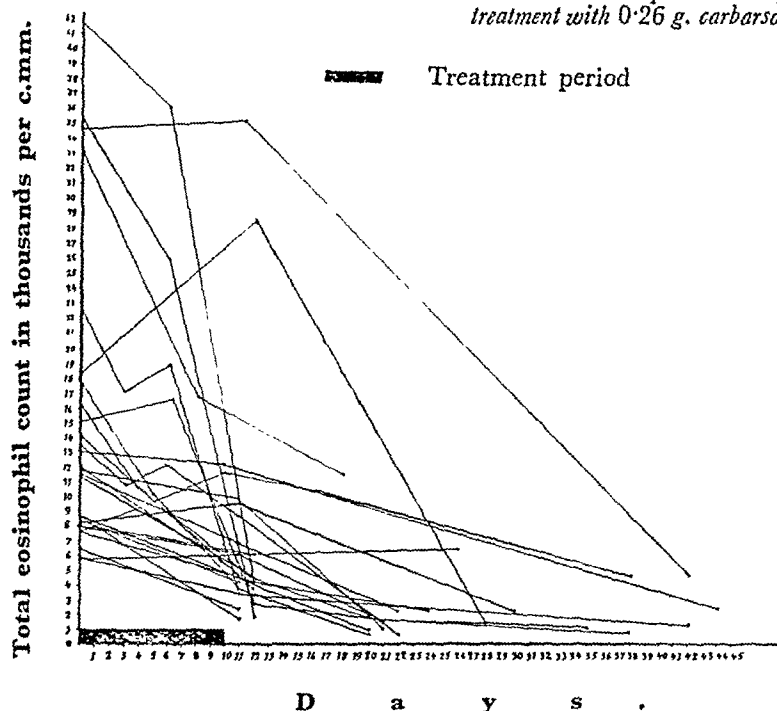
Four patients failed to show a satisfactory response to the treatment (Graph 2). One of these patients had persistent eosinophilia even after one year. Two patients reported after one year, with relapse. One patient had temporary aggravation of symptoms during the first few days of treatment.

The majority of the patients reached an eosinophil count below 2,500 by fourth week. The mean fall in the total eosinophil count during the critical period was

43 per cent with a standard deviation of 40 and standard error of 8 (Table II). Only 11 patients achieved a fall of more than 50 per cent during this period (Table III).

GRAPH 2.

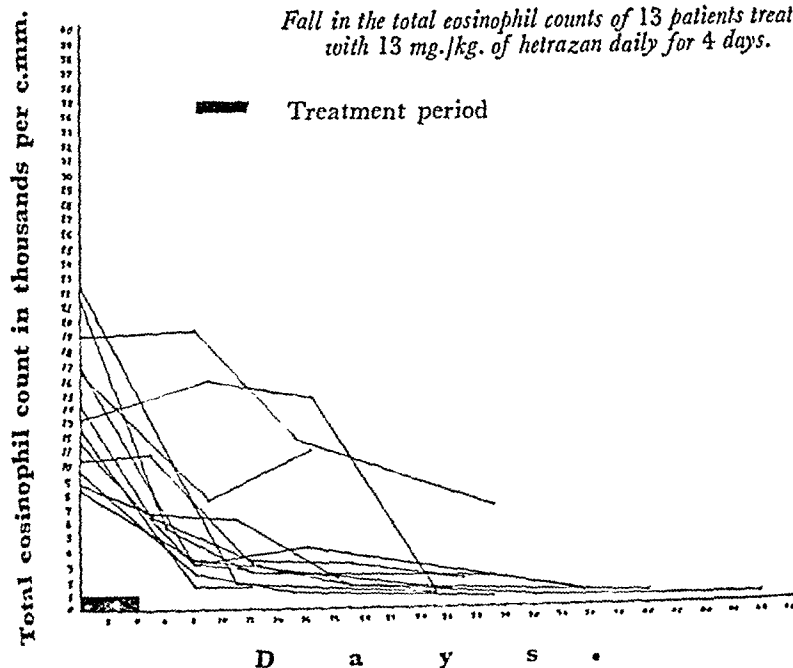
Fall in the total eosinophil counts of 27 patients after treatment with 0.26 g. carbarsone twice daily.



III. Group treated with 13 mg./kg. of hetrazan daily for 4 days.—Thirteen patients received the above dose of hetrazan for 4 days (Graph 3).

GRAPH 3.

Fall in the total eosinophil counts of 13 patients treated with 13 mg./kg. of hetrazan daily for 4 days.



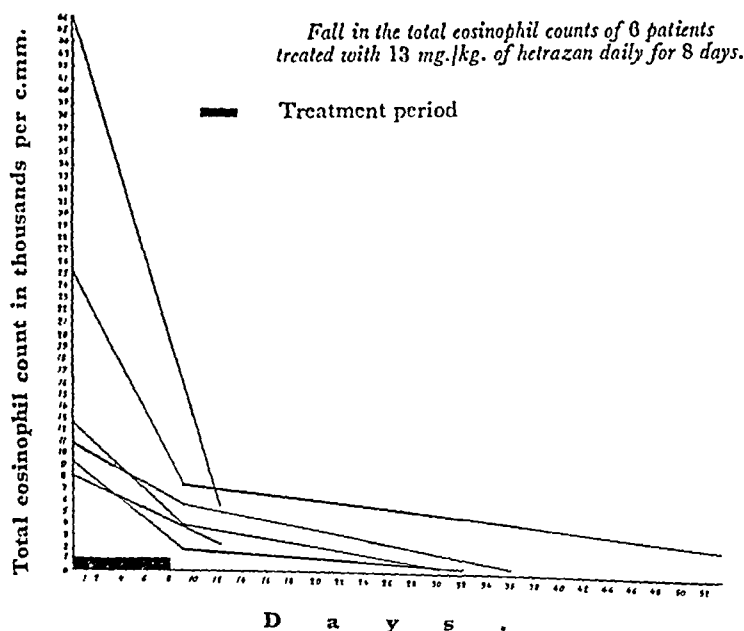
Eleven patients were adjudged to have been cured. Two patients showed an unfavourable response. One of them developed a pleural effusion during the course of the treatment, and the other showed a relapse of symptoms and rise in the eosinophil count after an initial improvement (Graph 3). One patient came back with a relapse after 10 months.

Aggravation of symptoms for few days earlier during the course of the treatment was noticed by three patients.

The mean fall in the eosinophil count during the critical period was 58 per cent, with the standard deviation of 36 and standard error of 10 (Table II). More than 50 per cent fall was found in 10 patients (Table III).

IV. *Group treated with 13 mg./kg. of hetrazan daily for 8 days.*—Only 6 patients were taken up for treatment with this dosage schedule (Graph 4). Further patients were not taken up in this group because our supply of hetrazan was limited and this series was consuming a large amount of hetrazan.

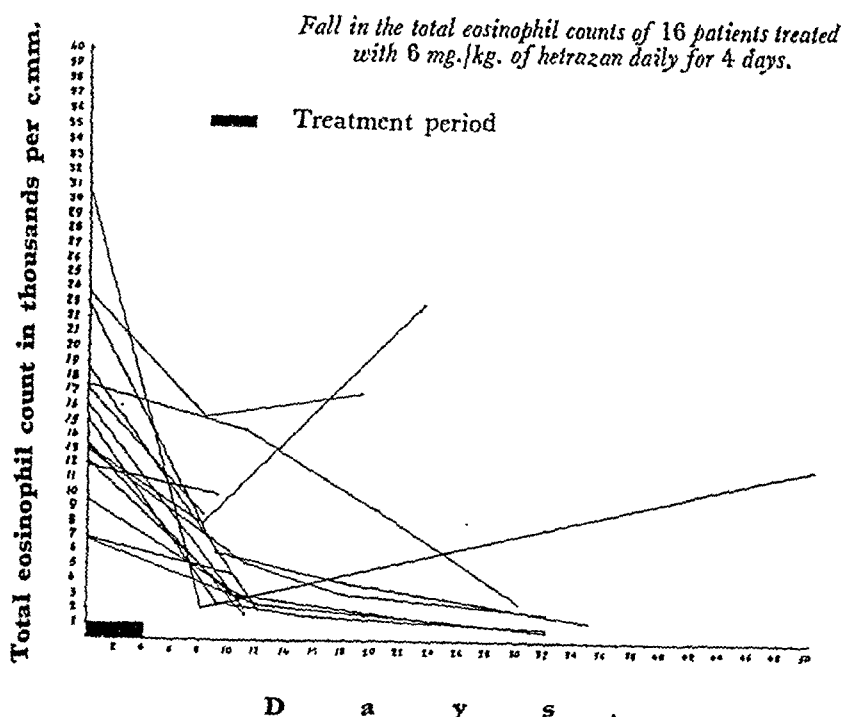
GRAPH 4.



All the patients in this group were completely cured during the follow-up period. The mean fall in the eosinophil count during the critical period was 71 per cent with a standard deviation of 13 and standard error of 5.5 (Table II). All except one of the patients had more than 50 per cent fall in the eosinophilia during this period (Table III).

V. *Group treated with 6 mg./kg. of hetrazan daily for 4 days.*—There were 16 patients in this group (Graph 5).

GRAPH 5.



The therapy was unsuccessful in 7 patients. Three of these patients showed a good response during the critical period, but thereafter their condition deteriorated. Initial aggravation of symptoms was seen in only one case.

The mean fall in the eosinophil count during the period between 8th and 12th day was 61 per cent with a standard deviation of 25 and standard error of 6 (Table II). More than 50 per cent fall during this period was seen in 10 patients (Table III). Out of these 10, 2 subsequently showed a significant rise of eosinophil level.

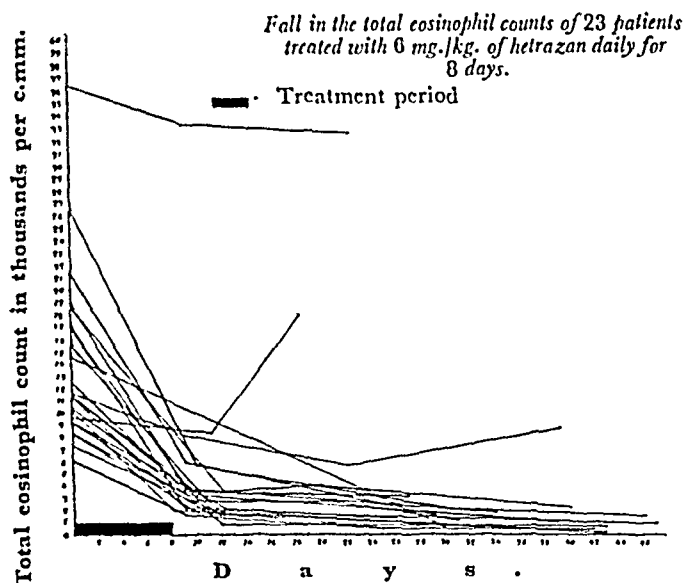
VI. *Group treated with 6 mg./kg. of hetrazan daily for 8 days.*—Twenty-three patients were taken up for trial with this schedule (Graph 6).

Failure to respond to treatment was observed in 3 patients. Two of these were, afterwards, treated with carbarsone and the third patient with a similar second course of hetrazan, and all of them showed favourable results. None of the patients experienced aggravation of symptoms during the earlier part of the treatment.

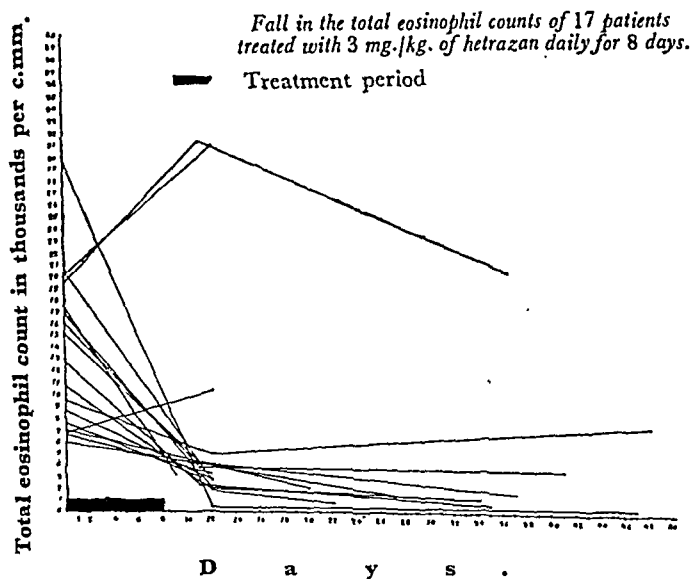
Graph 6 shows that majority of the patients had their eosinophil count lower than 2,500 soon after the end of the treatment. Mean fall of eosinophilia during the critical period was 71 per cent, with a standard deviation of 22 and standard error of 4.7 (Table II). Only 3 patients did not have a fall of more than 50 per cent during this period (Table III).

VII. *Group treated with 3 mg./kg. of hetrazan daily for 8 days.*—Seventeen patients were included in this group (Graph 7).

GRAPH 6.



GRAPH 7.

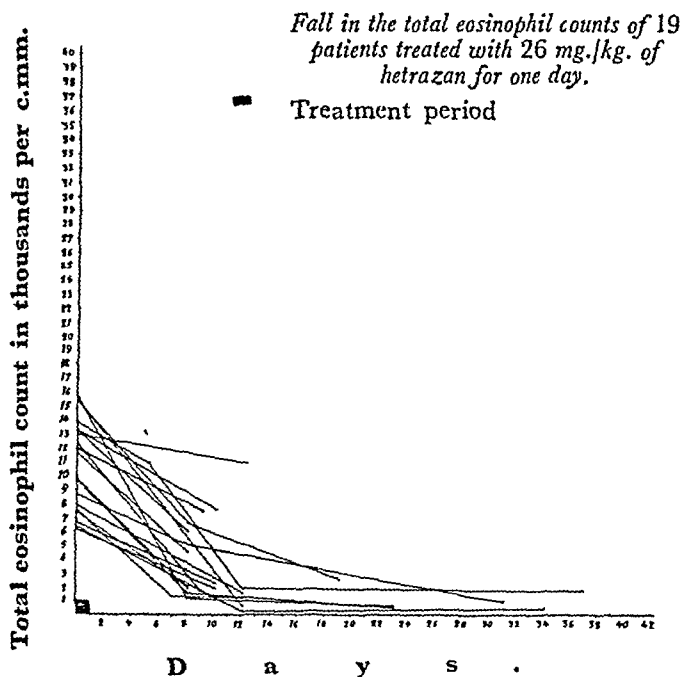


Four patients did not show any improvement with this treatment. Instead of significant improvement, 3 of these patients became worse after treatment. Good results were seen when two of the patients from this failure group were retreated with a second course of hetrazan.

Mean fall in the total eosinophil count during the critical period was 51 per cent, with a standard deviation of 54 and standard error of 13.5 (Table II). Eleven patients had a fall of more than 50 per cent during this period (Table III).

VIII. *Group treated with 26 mg./kg. of hetrazan for one day.*—26 mg./kg. of hetrazan was administered in three divided doses for only one day in 19 patients (Graph 8).

GRAPH 8.



Three patients did not show a good response to this regime. However, it was likely that two of them would have shown good response if the follow-up could have been extended.

All the patients in this group experienced aggravation of their symptoms on the day following the treatment.

The mean fall in the eosinophilia during the critical period was 69 per cent, with standard deviation of 22.8 and standard error of 5.2 (Table II). Less than 50 per cent fall was observed during this period in only 4 patients (Table III).

Examination of stools.—This was done in first 40 consecutive patients treated with hetrazan. In 24 patients no ova or parasites were seen in the stools. Ova of roundworms were seen in only 6 patients, of hookworm in 3 patients, of thread worm in 2 patients and giardiasis was observed in 5 patients.

COMMENTS.

All the patients presented in this series had a uniform clinical pattern. In the literature concerning tropical eosinophilia, a few cases with widely different symptomatology have been described (Chaudhuri *et al.*, *loc. cit.*) but as a general

rule the majority of patients conform to a definite clinical entity. It is possible to suspect this condition from clinical examination alone.

The mode of onset of this disease is insidious. Paroxysmal bouts of cough with scanty mucoid expectoration is the chief complaint. After such a bout, difficulty in breathing is felt by a large number of patients. This may be experienced as a suffocating sensation in the chest or as marked respiratory distress. Frequency and severity of the paroxysms of the cough are accentuated at night. This accentuation is so characteristic of the disease, as to be of diagnostic significance. Lack of sleep causes great weariness and desperate desire for rest. Patients having considerable nocturnal distress not infrequently remain comfortable and comparatively free from symptoms during the day. Constitutional symptoms, such as malaise, lassitude, anorexia, etc., are common. In a chronic case loss of weight is noticed. Mild pyrexia is described for few days at the onset or irregularly for a day or so during the course of the disease. Scarcity of physical signs is striking. Absence or presence of physical signs cannot be correlated with the severity of symptoms. When present, physical signs are usually confined to the lungs, and are perceived mainly on auscultation. They usually consist of prolonged expiration with sibilant and sonorous rhonchi scattered all over or heard in a localized area.

Even though the clinical picture is fairly definite, this criteria is highly subjective. The gradation of the symptoms is likely to be variable according to the bias of either the patient or the physician. Very little importance is given to symptoms in the evaluation of therapeutic results in the present work. Emphasis is placed mainly on the total eosinophil count, which is a definitive and objective criterion, and, hence, comparison of results in different groups is likely to be sound.

Total eosinophil counting by the modified Pilot's method is a more reliable index of the number of circulating eosinophils than the usual differential counting. No linear correlation seemed to exist between the level of eosinophilia and the severity of symptoms, but for a particular individual, fall in his initial eosinophil level was always associated with improvement in symptoms.

Ganatra and Lewis (unpublished) had administered aminopterin, one of the potent anti-folic acid drugs, to six patients with tropical eosinophilia. A fall of more than 50 per cent in the total eosinophil count was noted in each patient after giving a total dosage bordering on the toxic level. The symptomatic relief was significantly marked at this stage in all but one case. In all the patients, as soon as the drug was withdrawn, re-appearance of the symptoms and a rapid rise in the eosinophil counts were noted. Similar results have been observed with the use of ACTH and cortisone (Chaudhuri, 1951; Chaudhuri *et al.*, *loc. cit.*; and Reeder and Goodrich, *loc. cit.*). A precipitous fall in the eosinophil count with prompt remission of symptoms has been demonstrated after these drugs, but a rapid relapse to pre-treatment conditions shortly after stoppage of therapy was noted in many patients. The most illuminating fact that stands out, is that the reduction in the eosinophils was invariably associated with a clinical improvement. Response to both aminopterin and adrenal cortical hormones suggests that the symptomatology of this disease is mainly conditioned by the degree of eosinophilia, and for temporary clinical improvement, eradication of the primary disturbance is not necessary.

From the above data, we were led to an assumption that the fall in the eosinophilia is the surest guide to judge the improvement in a patient under treatment, and hence all the statistical considerations as regards to comparisons between different groups are based entirely on this sole factor.

The prime object of the present work was to determine the dose and evaluate the immediate effectiveness of hetrazan in the treatment of tropical eosinophilia. Different groups of patients were treated with different dosages of hetrazan and the results in each group were compared with the results of two control groups, one kept without treatment and the other treated with carbarsone. The results in different groups treated with hetrazan were also compared amongst themselves. These comparisons gave an idea of the absolute and the relative efficacy of hetrazan respectively. This investigation tries to establish the immediate effectiveness of hetrazan in tropical eosinophilia. As the follow-up period in individual cases is not long enough, no conclusion as regards to long-term effects on the frequency of relapses could be made. No comparison between parenteral arsenic and hetrazan was attempted, because it was not considered wise to treat a relatively benign condition like tropical eosinophilia with such a potentially toxic drug (Mehta, 1955).

As explained before, allocation of patients to different treatment groups was made at random. It was necessary to establish that the different treatment groups were comparable with each other, i.e., whether the randomization was effective or not. The pretreatment total eosinophil count was taken as an objective index to each patient, and the mean eosinophil count of the groups were compared (Table I).

TABLE I.

Group.				Number of patients in the group.	Mean eosinophil count per c.mm.
I.	No treatment	...	...	30	15,600
II.	Carbarsone	...	...	27	15,230
<i>Hetrazan</i> :—					
III.	13 mg./kg. daily for 4 days	...	...	13	14,070
IV.	13 mg./kg. daily for 8 days	...	...	6	18,600
V.	6 mg./kg. daily for 4 days	...	...	16	15,440
VI.	6 mg./kg. daily for 8 days	...	...	23	14,060
VII.	3 mg./kg. daily for 8 days	...	...	17	13,560
VIII.	26 mg./kg. for one day	...	...	19	10,860

Group Nos. I, II, III, V, VI, VII are well comparable and no significant difference exists in the mean eosinophil counts of these different groups. Group IV comprised of very few patients and the mean eosinophil count is higher than in the other groups. Hence, comparison of this group with the other groups is not likely to be valid. Group VIII has a statistically significant lower mean eosinophil count but the difference is small and comparison with other groups is likely to be valid.

Actions of hetrazan in filariasis are too well known to be enumerated here. Recently, it was found to have an action against intestinal nematodes as well.

Ascaris lumbricoides and *enterobius* are affected by its administration (Drill, 1954). This action on the intestinal nematodes is supposed to be fundamentally different from the action on the microfilariae and depends on the presence of the piperazine ring. A vain search for microfilariae in the blood of patients suffering from tropical eosinophilia has been made by several workers (Chaudhuri *et al.*, *loc. cit.*). When we accidentally found the effectiveness of this drug in tropical eosinophilia, we thought it probable that this effect might be due to its action on intestinal parasites. Loeffler, while describing a similar syndrome, had incriminated roundworms as the cause of the massive peripheral eosinophilia (Chaudhuri *et al.*, *loc. cit.*). Findings on stool examination of the first 40 patients in the present series did not lend support to this contention. More than half of these patients had no demonstrable parasite in their intestinal tract. Only 6 patients had ascariasis. Irrespective of the presence or absence of a helminth in the stools, all these patients showed a good response to the administration of carbarsone or hetrazan.

If left untreated, a patient of a tropical eosinophilia is likely to have frequent periods of remissions. To study the likelihood of improvement in symptoms coinciding with the natural period of remission, a control group of 30 patients was kept without any specific treatment. All the patients were followed beyond one week. Only three patients had remission, and even out of these three only one reached an eosinophil count lower than 2,500. The rest of the 27 patients failed to improve without treatment. The rate of failure, thus, is significantly very much higher as compared to the treatment groups. The mean fall in the eosinophil count was only 11 per cent, and this is much less than in the treated groups. The number of patients having more than 50 per cent fall in the eosinophil count during this period is also significantly low. It can be concluded that it is highly unlikely that a majority of patients having the classical symptoms of tropical eosinophilia and with a total eosinophil count above 5,000, will improve without any treatment within the short period of a fortnight although this may happen in one out of ten cases.

Patel (*loc. cit.*) had observed that oral treatment with carbarsone was as effective as the parenteral arsenic and, moreover, it was easier to administer. From a group of 27 patients, all except four showed a satisfactory response to this drug. When compared with the previous control group, the effectiveness of the drug stands undoubted. The improvement of the symptoms and the fall in the count were seen soon after the end of the 10 days' course of treatment. Initial aggravation of symptoms was observed in only one case, and it did not seem to be as frequent as when a large dose of hetrazan was used. The mean fall of 43 per cent during the critical period is significantly much higher than the no treatment group, but is significantly lower than in the groups treated with hetrazan in the dose of 13 mg./kg. daily for 8 days, 6 mg./kg. daily for 8 days or 26 mg./kg. for a day, and it is on par with the groups treated with other doses of hetrazan. The same holds true as regards the number of patients having more than 50 per cent fall in the eosinophil count during the critical period.

Hetrazan, 13 mg./kg. daily for 4 days, was the first dose schedule tried in present series. This dose was selected because a similar dose is used in the treatment of ascariasis (Drill, *loc. cit.*). Two patients, out of the total group of 13 were considered as failures. One of them had developed a pleural effusion during the

course of the treatment. This is a highly unusual complication. In his series of 207 patients, Vishwanathan (*loc. cit.*) met with this complication in only one case. The number of failures is not significantly less than that obtained with carbarsone, or with any other group treated with hetrazan. Temporary aggravation of symptoms initially in the course of the treatment was perhaps more frequent than that in the Carbarsone group. The mean fall in the eosinophil count was 58 per cent, and 10 patients had more than 50 per cent fall during the critical period. These figures are in no way statistically better than those obtained with carbarsone. This dose was as effective as carbarsone, but in no way better.

A dose of 13 mg./kg. of hetrazan daily for 8 days was tried in only 6 patients for the reasons previously mentioned. All the patients in this group showed a favourable response to the treatment. The mean fall in the eosinophil count during the critical period was 71 per cent, and 5 patients had more than 50 per cent fall during this period. All these factors appear to suggest that this dose schedule is better than the carbarsone group and the previous group treated with the same dose for half the number of the days, but the number of patients in this group is so small that statistical conclusions are not valid.

TABLE II.

Comparison of mean per cent fall in the total eosinophil count during the critical period.*

Group.	Mean fall, per cent.	Standard deviation.	Standard error.	Statistical evaluation as compared to no treatment group.	Statistical evaluation as compared to carbarsone group.
No treatment (28)† ...	11	46	9	...	Significantly less fall.
Carbarsone (25) ...	43	40	8	Significantly more fall	...
<i>Hetrazan:—</i>					
13 mg./kg. daily for 4 days (13) ...	58	36	10	„ „	No significant difference.
13 mg./kg. daily for 8 days (6) ...	71	13	5.5	„ „	Significantly more fall.
6 mg./kg. daily for 4 days (16) ...	61	25	6	„ „	No significant difference.
6 mg./kg. daily for 8 days (22) ...	71	22	4.7	„ „	Significantly more fall.
3 mg./kg. daily for 8 days (16) ...	51	54	13.5	„ „	No significant difference.
26 mg./kg. for 1 day (19)	69	23	5.3	„ „	Significantly more fall.

*A few patients on whom observations were not obtained during the critical period are omitted for this statistical consideration.

†Indicates number of patients in each group.

Seven patients out of a total of 16 in the group failed to respond to a dose of 6 mg./kg. of hetrazan daily for 4 days. The rate of failure is, still, much less than the control group without treatment. Initial aggravation of the symptoms was infrequent. The fact that only 10 patients had more than 50 per cent fall in the

total eosinophil count and the mean fall in the eosinophil count of 61 per cent, proves that the drug is in no way better than carbarsone.

TABLE III.

Comparison by chi-square method of the number of patients having more than 50 per cent fall in the total eosinophil count during the critical period.*

Group.			Number of patients in the group.	Number of patients having more than 50 per cent fall in the eosinophil count.	Statistical evaluation as compared to no treatment group.	Statistical evaluation as compared to carbarsone group.
No treatment	...	...	28	3	...	Significantly less fall.
Carbarsone	...	...	25	11	Significantly more fall	...
<i>Hetrazan:—</i>						
13 mg./kg. daily for 4 days	...	...	13	10	" "	No significant difference.
13 mg./kg. daily for 8 days	...	...	6	5	" "	Significantly more fall.
6 mg./kg. daily for 4 days	...	...	16	10	" "	No significant difference.
6 mg./kg. daily for 8 days	...	...	22	19	" "	Significantly more fall.
3 mg./kg. daily for 8 days	...	...	16	12	" "	No significant difference.
26 mg./kg. for 1 day	...	...	19	15	" "	Significantly more fall.

*A few patients on whom observations were not obtained during the critical period are omitted for this statistical consideration.

TABLE IV.

Comparison by chi-square method of successes and failures in different groups.

Group.			Number of patients in each group.	Number of failures.	Number of successes.	Statistical evaluation as compared to no treatment group.	Statistical evaluation as compared to carbarsone group.
No treatment	...	...	30	27	3	...	Significantly more failures.
Carbarsone	...	...	27	3	24	Significantly fewer failures	...
<i>Hetrazan:—</i>							
13 mg./kg. daily for 4 days	...	...	13	2	11	" "	No significant difference.
13 mg./kg. daily for 8 days	...	...	6	0	6	" "	" "
6 mg./kg. daily for 4 days	...	...	16	7	9	" "	" "
6 mg./kg. daily for 8 days	...	...	23	3	20	" "	" "
3 mg./kg. daily for 8 days	...	...	17	4	13	" "	" "
26 mg./kg. for one day	...	...	19	3	16	" "	" "

However, when the same dose was given for 8 days, the number of patients failing to respond to treatment was significantly less. The mean fall in the

eosinophil count during the critical period was 71 per cent, and 19 patients had more than 50 per cent fall. These results show that in this dose schedule hetrazan is significantly better than carbarsone at a dose level of 0.52 g. daily for 10 days.

Hetrazan in a dose of 3 mg./kg. for 8 days, showed 4 failures to treatment out of 17 patients. Mean fall during the critical period was 41 per cent and 12 patients had more than 50 per cent fall. This regime gives results comparable to those obtained with carbarsone.

A large single dose of hetrazan has been tried in the treatment of filariasis. (Laurie, 1949). On this basis, it was decided to try a single dose of 26 mg./kg. of hetrazan. Single doses larger than this have been tried in the treatment of filariasis without any untoward effects. (Laurie, *loc. cit.*). When the above dose was given to few patients of tropical eosinophilia, a severe but transitory reaction developed in all patients. The reaction consisted of fever, marked asthmatic attack and vomiting, and was similar to the Herxheimer reaction noticed in the treatment of syphilis. The same daily dose was then, divided into three portions and was administered at 4 hours intervals to a group of 19 patients. There were only three treatment failures in this group, mean fall of eosinophil level during the critical period was 69 per cent, and less than 50 per cent fall was seen in only 4 patients. This suggests that such a regime had better results than the carbarsone group. Slight temporary aggravation of symptoms was still noticed in all the patients who were administered 3 doses. The effectiveness of this single day treatment is surprising.

It is a well-known fact that this disease is likely to recur even a long period after successful treatment. In the present series, 3 patients came with a relapse after a lapse of 10 to 12 months.

There is no doubt that hetrazan was uniformly effective in lowering the total eosinophil count and in producing symptomatic cure in the majority of the patients in the present series. Almost all the dosage schedules of hetrazan tried were found to be effective, but minor variations were seen in the degree of effectiveness.

The number of patients failing to respond to treatment in the carbarsone group and in the different hetrazan groups was not significantly different. Relief of symptoms was usually earlier with hetrazan despite the fact that temporary aggravation of symptoms early in the course of the treatment was more frequent when high doses of hetrazan were employed.

About 12 years after the first report of the usefulness of organic arsenicals, another drug is found to have equal, if not better, therapeutic response. The effectiveness of hetrazan in reducing eosinophilia associated with proved filarial infection has been noted in the past by various workers (Faure, Geyer and Gueffier, 1955; Freiss, Pierrou and Segalen, 1953; and Galliard and Lariviere, 1953).

Three dose schedules of hetrazan, namely 13 mg./kg. daily for 8 days, 6 mg./kg. daily for 8 days and 26 mg./kg. daily for one day, produced a better fall in the eosinophil counts during the critical period. As compared to carbarsone, earlier recovery towards normal is possible with the above regimes of hetrazan. The group treated with 26 mg./kg. for a day had a low mean eosinophil count initially, suggesting that randomization in this group was not as effective as in

other groups. Hence, better effectiveness of the drug in this group is open to doubt. Moreover, initial aggravation of symptoms during the treatment was distressing to some of the patients. A dose of 6 mg./kg. of hetrazan daily for 8 days can be recommended as giving better results than carbarsone at a dose of 0.52 g. daily for 10 days.

No definite data is available as regards to the toxic effects of arsenic in this condition. Many of the cases of arsenical encephalopathy or myelopathy described in recent times in India were after the use of parenteral arsenic in tropical eosinophilia (Mehta, *loc. cit.*). Use of arsenic in any form is always associated with potential hazards. Hetrazan is known to be non-toxic in the doses which we have employed and even minor side-effects were not observed in the patients included in the present series.

SUMMARY.

1. Results of treatment in 94 consecutive patients of tropical eosinophilia, treated with different dosages of hetrazan are recorded. These results are compared with results obtained in two control groups. The first control group consisted of 30 patients who were kept without any specific treatment, the second control group comprised of 27 patients who were treated with 0.26 g. of carbarsone twice daily for 10 days. Hetrazan was tried in the dose schedules of 13 mg./kg. daily for 4 days, 13 mg./kg. daily for 8 days, 6 mg./kg. daily for 4 days, 6 mg./kg. daily for 8 days, 3 mg./kg. daily for 8 days and 26 mg./kg. for one day.

2. The results of treatment in all the patients treated with hetrazan compared favourably with the first control group, kept without specific treatment.

3. The rate of treatment failure with hetrazan in the above various dosages was not significantly less than carbarsone, but significantly earlier and more fall in the eosinophil count was seen in three groups treated with hetrazan, 13 mg./kg. for 8 days, 6 mg./kg. for 8 days and 26 mg./kg. for one day.

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STUDIES ON D860—ANOTHER NEW ORAL HYPOGLYCEMIC SULFONAMIDE.

BY

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AND

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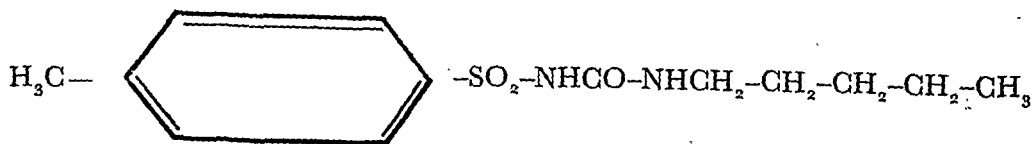
(From the Central Drug Research Institute, Lucknow.)

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THE discovery of the hypoglycemic property of sulphonylurea (BZ-55) by Achelis and Hardebeck (1955), Franke and Fuchs (1955) and Bertram, Benfeldt and Otto (1955) and its confirmation from independent sources (Kinsell *et al.*, 1956; Kirtley *et al.*, 1956; and Unger and Davidson, 1956), almost proved the usefulness of this compound as an oral remedy for diabetes. Ability of BZ-55 to exert satisfactory control of elderly diabetics on long continued administration and the apparently non-toxic nature of the drug, even on uninterrupted prolonged administration (Franke and Fuch, *loc. cit.*; Ridolfo and Kirtley, 1956; Kinsell *et al.*, *loc. cit.*) have further evoked a general interest in these sulfonamides. Recently, a group of English scientists experienced different degrees of toxicity while using BZ-55 in diabetic patients (Walker *et al.*, 1956; Wolff *et al.*, 1956; and Duncan *et al.*, 1956). In view of the alleged toxicity of BZ-55, it is desirable to obtain relatively non-toxic sulfonamides which are at the same time hypoglycemic in nature.

D860, called Rastinon by trade name, has been claimed to be such a compound having a chemical composition of N- (4-methyl-benzene-sulphonyl)-N'-butyl urea.

It has the following structural formula:—



Scholz and Bander (1956), Mohnike and Stotter (1956), Stotter *et al.* (1956), Bander and Scholz (1956) have investigated on different aspects of pharmacological action of D860 including its hypoglycemic property and toxicity studies. From their results it appears that the compound is practically non-toxic and apparently more potent than BZ-55 in inducing hypoglycemia. Mohnike and Stotter (*loc. cit.*) and Mirsky *et al.* (1956) have also recommended its use in selected groups of diabetic patients.

In order to determine the suitability of prolonged use of the new hypoglycemic sulfonamide in clinical diabetes the following experiments were undertaken. The cause of hypoglycemia being not known, attempt has also been made to find out the nature of the hypoglycemic action of D860.

METHODS AND MATERIALS.

Albino rats of C.D.R.I. colony, weighing approximately 150 g. to 200 g., were kept on a stock diet for 7 days and finally 120 animals were selected for our experiment.

First group (group I) of 24 animals was sub-divided into 3 batches of 8 each. All the animals were kept without food (water allowed *ad lib.*) for 12 hours. After collection of their blood for sugar estimation the three batches of animals were fed with three different doses of D860. Thus, each of the first, second and third batch of animals were fed 0.25 g./kg., 0.5 g./kg., and 1.0 g./kg. of D860, respectively. Blood sugar was determined every hour after D860 feeding, for 5 hours. No food but water was allowed *ad lib.* during the period of test.

Second group (group II) of 25 animals was fed D860 in the dose of 0.5 g./kg./day each, for 28 days. Blood sugar (in 12 hours fasting condition) of all the animals was determined once a week during 28 days of D860 feeding and their weight was recorded.

Third group (group III) of 16 animals was divided into 2 batches of 8 each. One batch of 8 animals served as untreated control and the other batch of 8 animals was fed D860 in the dose of 0.5 g./kg./day each for 28 days. Two days (48 hours) after discontinuation of D860 feeding, insulin-sensitivity test was performed on the D860-fed batch as well as on untreated control batch of animals. Animals of both the batches were kept without food for 12 hours prior to and during insulin-sensitivity test. Out of 48 animals of fourth group (group IV), 16 animals were fasted for 16 hours and glucose-tolerance test was performed on them. The remaining 32 animals were divided into 4 batches of 8 animals each. First three batches were fed with three different doses of D860 exactly in the same way as stated for the three batches of group I animals. Fourth batch was also fed D860 exactly in the same way as stated for group II animals. Glucose-tolerance test was performed on the first three batches of animals 2 hours after D860 feeding and on the fourth batch of animals after 48 hours of the last dose of D860.

Last group (group V) of 24 animals was divided into three batches of 8 each. First batch of animals was starved for 12 hours (water allowed *ad lib.*) and their blood collected by heart puncture for cholesterol and glutathione (reduced) estimation. The animals were then decapitated and exsanguinated and a portion of

their liver was weighed out as quickly as possible for glycogen estimation. Another portion of liver was weighed out for cholesterol estimation. Second batch of animals under similar fasting and condition was fed D860 in a single dose of 0.5 g./kg. each and 2 hours after D860 feeding their blood and liver were taken out exactly in the same way and for similar tests as in first batch of animals. Third batch of animals was fed D860 in the dose of 0.5 g./kg./day for 28 days. After two days (48 hours) of discontinuation of D860 feeding the animals were starved for 12 hours. Their blood and liver were also taken out exactly in the same way and for similar examination as in the case of other two previous batches.

Liver for cholesterol estimation was thoroughly macerated with washed sea-sand and extracted with ether-alcohol mixture. Blood sugar was estimated by Folin and Wu (1920) micromethod. Blood and liver cholesterol was determined by the method of Bloor (1916). Liver glycogen was determined by the method of Good *et al.* (1932). Blood glutathione (reduced) was determined by the method of Woodward and Fry (1932). Insulin-sensitivity test was performed by the method of Root Ellis and Staub (1954). Insulin used was 0.3 μ /kg. of body-weight. Glucose-tolerance test was carried out according to the method of Cole and Harned (1938). Glucose used was 3.5 g./kg. of body-weight in 10 per cent solution.

RESULTS.

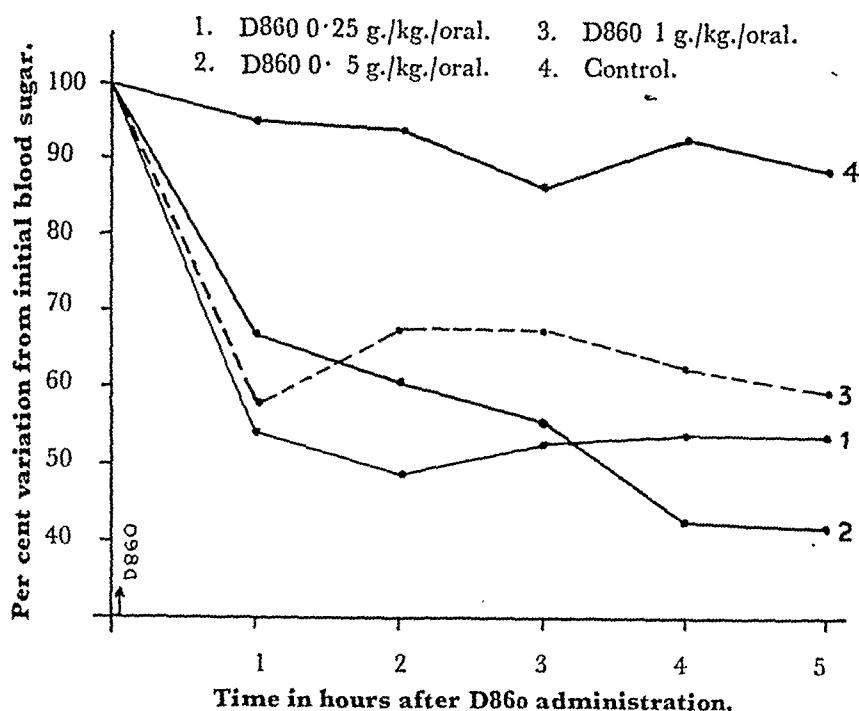
Effect of oral administration of three separate single doses of D860 on the blood sugar of albino rats has been presented in Table I and Graph 1. The results show that a single dose of 0.25 g./kg. of D860 lowers the blood sugar in albino rats by nearly 45 to 50 per cent within two hours of its administration and also maintain the same low level of blood sugar during the rest of the period of observation. When the dose of D860 is increased to 0.5 g./kg., it causes a progressive fall in the blood sugar level during the whole period of observation and the blood sugar is lowered nearly by 58 per cent at the end of 5th hour. Administration of the next higher dose of 1 g./kg. of D860 brings down the blood sugar nearly by 43 per cent (maximum fall) at the end of first hour. During the next hour

TABLE I.

Effect of feeding of a single dose of D860 (Rastinon) on blood sugar level in albino rats.

Dose of D860.	BLOOD SUGAR IN MG./100 C.C. (MEAN $\pm$ S.E.):					
	Before D860 (fasting).	After D 8 6 0 .				
		1 hour.	2 hours.	3 hours.	4 hours.	5 hours.
<i>Nil</i>	135 $\pm$ 2.6	128 $\pm$ 4.8	125 $\pm$ 6.22	113 $\pm$ 2.13	123 $\pm$ 1.33	112 $\pm$ 5.12
0.25 g./kg.	135 $\pm$ 2.6	78 $\pm$ 2.4	70 $\pm$ 3.2	74 $\pm$ 3.1	76 $\pm$ 3.7	75 $\pm$ 3.2
0.5 g./kg.	135 $\pm$ 2.6	87 $\pm$ 2.6	80 $\pm$ 3.6	73 $\pm$ 2.1	56 $\pm$ 4.8	54 $\pm$ 4.8
1 g./kg.	135 $\pm$ 2.6	79 $\pm$ 1.7	92 $\pm$ 2.8	92 $\pm$ 6.9	86 $\pm$ 3.5	81 $\pm$ 2.3

GRAPH 1.



(2nd hour after D860), however, blood sugar tends to rise and actually remains at a higher level during second and third hours than that of first hour. For the last two hours, though the blood sugar is coming down slowly and is lowered nearly by 40 per cent at the end of 5th hour, it still remains at a higher level than the corresponding levels of the two previous batches fed with lower doses. Thus, it appears from the above results that though all the three doses of D860 used in this experiment are capable of bringing down the blood sugar to different degrees in albino rats. 0.5 g./kg. dose has the most pronounced and prolonged action on blood sugar, whereas 1 g./kg. dose shows least effect than either of the other two lower doses used.

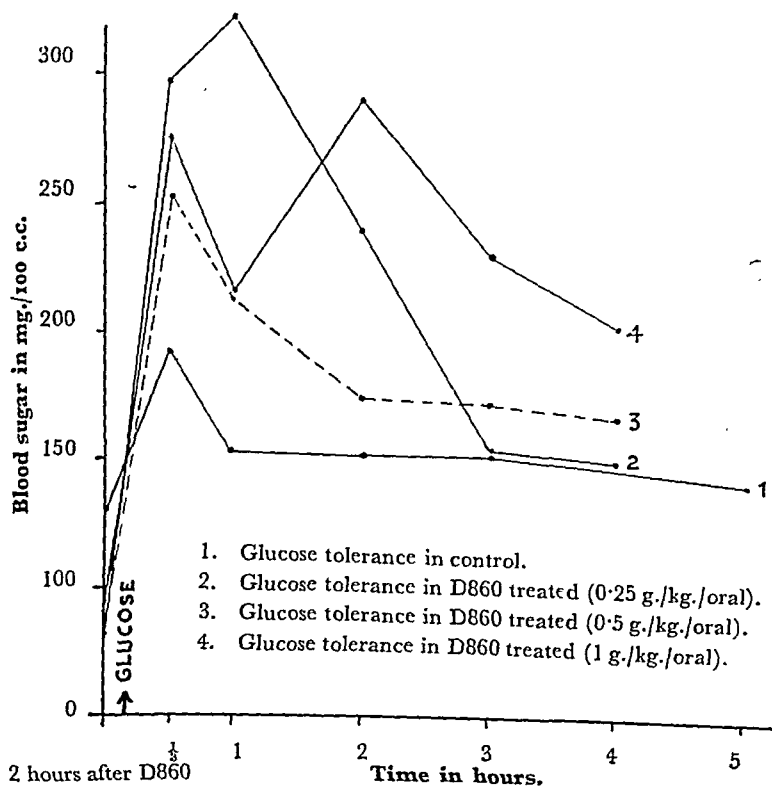
The results of glucose-tolerance test performed in albino rats: (1) 2 hours after oral administration of three separate single doses, and (2) 48 hours after daily oral administration of 0.5 g./kg./day dose of D860 for 28 days have been presented in Table II and Graphs 2 and 3.

In animals fed 0.25 g./kg. of D860, the blood sugar rises to a maximum level of 323 ± 21.8 mg. per cent, one hour after administration of glucose (instead of 1/2 hour after, when maximum rise is observed in normal control). Blood sugar thereafter goes on decreasing and comes down to a level of 154 ± 22.5 mg. per cent at the end, 5th hour after glucose administration. In animals fed next higher dose of 0.5 g./kg. of D860, maximum rise of blood sugar to a level of 253 ± 26.1 mg. per cent occurs 1/2 hour after glucose. For the rest of the period of observation blood sugar shows continuous fall and reaches a level of 169 ± 18.9 mg. per cent at the end of 5th hour. Variations in the blood sugar level after administration of glucose in animals fed 1 g./kg. of D860 is somewhat different. Blood sugar rises to a level of 278 ± 1.7 mg. per cent within 1/2 hour after glucose and is followed by a fall in

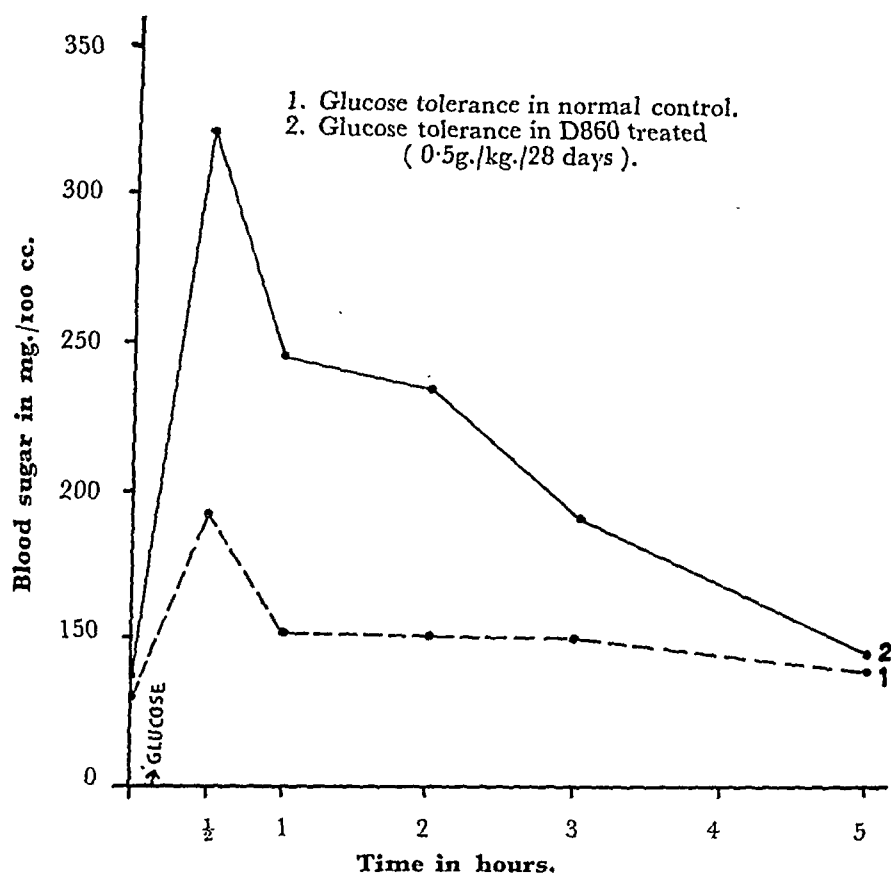
TABLE II.
Mean blood sugar values during glucose-tolerance test in normal and D860-fed rats.

Number of animal.	Fasting B.S. (mean $\pm$ S.E.) in mg./100. c.c.	Dose of D860 fed.	BLOOD SUGAR (MEAN $\pm$ S.E.) IN MG. PER 100 C.C.						
			2 hours after D860 feeding.	After administration of glucose					Glucosuria present.
				$\frac{1}{2}$ hour.	1 hour.	2 hours.	3 hours.	5 hours.	
16 (Normal)	136 ± 1.25	—	—	192 ± 3.6	153 ± 6.5	153 ± 6.4	153 ± 7.3	141 ± 6.3	in 6 out of 16
8	„	0.25 g./kg.	67 ± 3.3	298 ± 21.7	323 ± 21.8	240 ± 28.1	164 ± 23.7	154 ± 22.5	—
8	„	0.5 g./kg.	79 ± 1.5	253 ± 26.1	213 ± 29	175 ± 13.4	174 ± 21	169 ± 18.9	—
8	„	1 g./kg.	76 ± 1.3	278 ± 1.7	216 ± 19.7	290 ± 19.3	230 ± 34	202 ± 11.3	—
8	„	0.5 g/kg/day for 28 days	136 ± 1.4	323 ± 39	248 ± 52	236 ± 33	194 ± 19	156 ± 14	—

GRAPH 2.



GRAPH 3.



blood sugar level to 216 ± 19.7 mg. per cent during the next 1/2 hour (1 hour after glucose administration) but this fall is again followed by a rise in blood sugar level to 290 ± 19.3 mg. per cent (maximum) during the 2nd hour after glucose administration. Thereafter, blood sugar goes on decreasing and reaches a level of 202 ± 11.3 mg. per cent at the end of 5th hour. This double rise in the blood sugar level after glucose is not exhibited in the other two batches of animals fed with lower doses of D860. The results of glucose-tolerance test on animals fed 0.5 g./kg./day of D860 for 28 days are in some respect similar in nature to those of 0.5 g./kg. single dose fed animals. The maximum rise of blood sugar occurs within 1/2 hour after glucose and is followed by a continuous reduction in the blood sugar which comes down to a level of 156 ± 14 mg. per cent at the end of 5th hour. Therefore, it is clear that glucose-tolerance test in D860-fed rats differs from that in normal controls as follows:—

- (i) the maximum rise (peak values) of blood sugar after glucose is markedly higher (always beyond 0.200 per cent) than that in the normal control animals.
- (ii) The maximum rise of blood sugar does not always occur 1/2 hour after glucose administration.
- (iii) Blood sugar level at the end of 4th to 5th hour remains usually above the preglucose fasting level.
- (iv) Glycosuria in marked degree is present in all animals, whereas in normal control animals glycosuria is present only in 6 out of 16 animals.

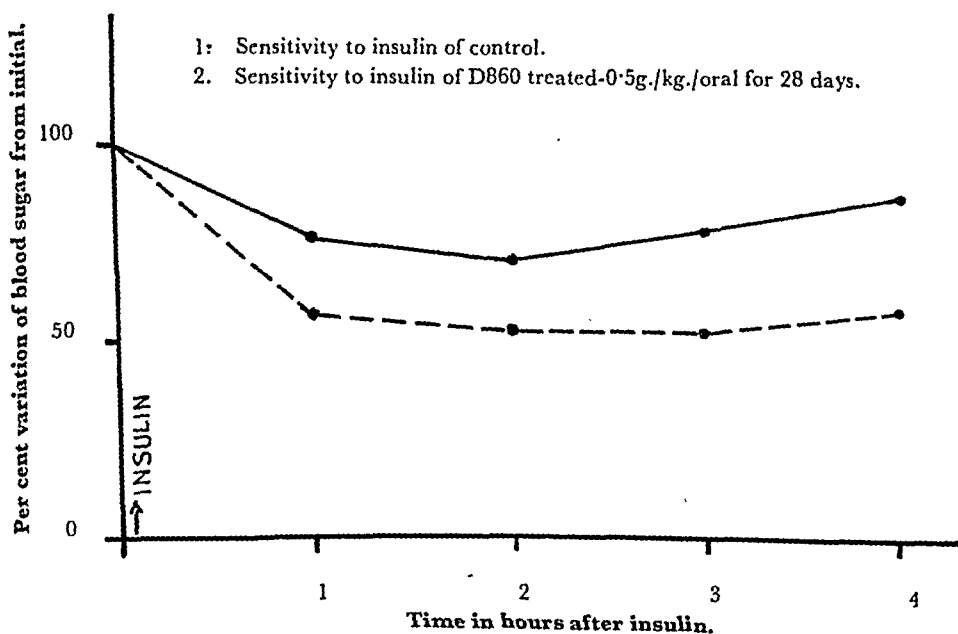
The results of insulin sensitiveness in normal control animals and in animals after 28 days feeding of D860 are represented in Table III and Graph 4. The results show that the maximum hourly fall of blood sugar in D860-fed animals after intramuscular injection of 0.2μ . insulin/kg. is 48 per cent as compared to 33 per cent in normal control, under similar conditions. At the end of 4 hours the blood sugar in D860-fed animals is still 44 per cent below, whereas in normal control it is only 15 per cent below the pre-insulin fasting level. Thus, it appears that, administration of D860 potentiates the action of exogenous insulin both in intensity and duration in albino rats.

TABLE III.

Effect of insulin (0.3μ /kg./SC) on blood sugar level of normal and D860-fed rats.

	BLOOD SUGAR IN MG./100 G.C. (MEAN $\pm$ S.E.):				
	Before insulin (feeding).	After administration of insulin.			
		1 hour.	2 hours.	3 hours.	4 hours.
Normal ...	134 ± 1.25	102 ± 2.32	93 ± 5.6	107 ± 1.92	114 ± 3.66
D860 feeding 0.5 g./kg. day for 28 days ...	136 ± 1.4	79 ± 6.8	71 ± 4.5	70 ± 6.7	76 ± 4.8

GRAPH 4.



The results of blood glutathione, blood and liver cholesterol and liver glycogen in normal control animals, in animals fed 0.5 g./kg. single dose of D860 and in animals fed 0.5 g./kg./day for 28 days have been shown in Table IV. It becomes apparent from the results that glutathione and cholesterol levels of the blood are not materially affected either by a single dose, 0.5 g./kg. of D860 or by its prolonged administration. Liver glycogen level appears to be definitely increased by administration of both single dose and repeated doses of D860. Liver-cholesterol values in D860-fed animals show slight increase over that of normal control. The increase is higher in animals fed D860 for 28 days. In no animal any loss in the body-weight has been observed following feeding of D860 for 28 days and there has been no mortality during the period of observation.

TABLE IV.

Effect of D860 feeding on blood glutathione; blood and liver cholesterol, and liver glycogen in albino rats.

	Normal.	D860 TREATED:	
		0.5 g./kg./oral/ single.	0.5 g./kg./oral/ 28 days.
Blood glutathione (reduced), mg./100 c.c.	38 $\pm$ 1.6	38 $\pm$ 1.8	36 $\pm$ 1.05
Blood cholesterol, mg./100 c.c.	100 $\pm$ 0.8	99 $\pm$ 2.0	95 $\pm$ 3.4
Liver cholesterol, mg./100 g. wet tissue	264 $\pm$ 18.33	321 $\pm$ 13.22	347 $\pm$ 7.6
Liver glycogen*, g./100 c.c. wet tissue	2.157 $\pm$ 0.21	2.279 $\pm$ 0.354	2.682 $\pm$ 0.11

*Expressed as glucose.

Mean average of 8 data in each case.

DISCUSSION.

From the data, it is clear that feeding of D860 in a single dose or in repeated doses does not alter the glutathione and cholesterol level of blood in albino rats. Liver glycogen and the liver cholesterol values show slight increase. Increased liver-glycogen value was also obtained by Bander and Scholz (*loc. cit.*) by using D860 in other species of animals. No toxic effect of the drug was observed during our experiment even when the drug was administered for a long time (28 days) in moderately high dose (0.5 g./kg.). This is apparent from the absence of loss of weight in the D860-treated animals and there being no mortality from its long continued use and no decrease in liver-glycogen level of the D860-fed animals during the period of observation.

The results of our experiment further show that oral administration of D860 brings down the fasting blood sugar level in albino rats. The findings are in agreement with the observations made by Scholz and Bander (*loc. cit.*) who obtained similar blood sugar lowering effect of the same sulphonamide on different species of animals. Though all the three doses of D860 used in our experiment apparently

cause lowering of the fasting blood sugar level, a detailed examination of the results reveal that there exists a difference so far as intensity and duration of the hypoglycemic activity of the three doses of D860 are concerned. The highest degree of fall (58 per cent) in the blood sugar level which is maintained for the longest period (up to 5 hours) is obtained by the intermediate dose of 0.5 g./kg. of D860, whereas when the highest dose of 1 g./kg. of D860 is used minimum fall (43 per cent) occurs. Even this fall in blood sugar level is short-lived which shows a tendency to rise within 5 hours. This indicates that there probably exists a relation between the limit of dose and maximum blood sugar lowering effect of the drug. The drug appears to exert its maximum blood sugar lowering effect at a certain dose level. Increase of the dose beyond this critical level may not cause any further lowering of the blood sugar level. On the other hand, this increase in the dose beyond the critical level may vitiate the effect, as is evident from results obtained by us. This is supported by the results obtained by Diengott and Mirsky (1956) who found that increase in the dose of sulfonylurea beyond a definite limit failed to increase the hypoglycemic potency of the drug.

The results of glucose-tolerance test, in all the animals whether fed single or repeated doses of D860 show a diabetic-like height of the peak values of blood sugar after glucose. Moreover, the return of the blood sugar to the pre-glucose fasting level is delayed beyond 4 to 5 hours. The reason or reasons for this abnormality are not fully clear. It may be due to temporary lack of availability of sufficient insulin as an effect of exhaustion of insular apparatus caused by the stimulating effect of previously administered D860 on β cells of the pancreas. Whatever may be its reason, the persistent diabetic-like peak value and the delay in the return of blood sugar to the pre-glucose level within 5 hours after glucose injection is a matter of investigation before the place of hypoglycemic sulphonamides in the treatment of *Diabetes mellitus* is established.

Our results show that the same dose of insulin causes greater and sustained fall of blood sugar level in D860-fed animals as compared to the effect on normal control. Enhanced hypoglycemic action of insulin by other hypoglycemic sulphonamides drugs has been observed by Houssay and Migliorini (1956), hypoglycemic sulphonamides when administered with insulin, has been shown by Macallum and Sivertz (1942) to accelerate the hypoglycemic action of insulin resulting in convulsion of animals. The increased hypoglycemic action of insulin in D860-fed animals may be due to the combined action of exogenous and endogenous insulin, the later being liberated by the stimulating action of D860 on the β cells of the pancreas. Enhanced and prolonged action of insulin may also be due to the inhibition of insulin-destroying enzyme insulinase by D860 (Mirsky *et al.*, 1956) or it may be due to both of the above-mentioned action of D860 (Diengott and Mirsky, *loc. cit.*).

The results of glucose-tolerance test and insulin-sensitivity test obtained by us support the view that hypoglycemic effect of sulphonamide D860 is related to the mechanism of secretion of insulin and its utilization. The early hypoglycemia is due to rapid liberation of insulin from Beta cells and the sustenance of the hypoglycemic response is probably due to inhibition of insulinase enzyme.

SUMMARY.

Effect of D860 (Rastinon) Hoechst, a hypoglycemic sulphonamide, on fasting blood sugar level, on glucose-tolerance test, on insulin-sensitivity test and on cholesterol and glycogen level in liver, and cholesterol and glutathione level in blood of albino rats has been studied.

(1) Oral administration of D860 in three dose (0.25 g./kg., 0.5 g./kg. and 1 g./kg.) lowers the fasting blood sugar level. The maximum and prolonged hypoglycemia is produced by a dose of 0.5 g./kg.

(2) Administration of both single and repeated doses of D860 cause a diabetic-like peak value in the glucose tolerance curve and delays the return of blood sugar to preglucose level within 5 hours.

(3) D860 feeding increases the effect of insulin both in intensity and duration.

(4) Blood glutathione and cholesterol level in D860-fed animals remain normal. Slight increase in glycogen and cholesterol content of the liver is observed.

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EFFECT OF CHLORAMPHENICOL ON WEANLING RATS*.

A STUDY OF CHANGES IN THE BLOOD AND THE RATE OF GROWTH.

BY

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PHARMACOLOGIC and toxic effects of chloramphenicol have been studied in a number of animal species (Glazko, 1949; Gruhzt, 1949). Recently, this antibiotic has been reported as causing abnormal hæmatologic reactions in man following its administration in therapeutic doses (Lewis *et al.*, 1952; Hodgkinson, 1954). It is not known whether chloramphenicol *per se* or one of its metabolites produced in the human body is responsible for these abnormal hæmatologic manifestations. Chloramphenicol and one of its congeners have been fed to young rats in varying concentrations of the diet in an attempt to reproduce the syndrome clinically observed in man. To study whether these substances were toxic to the hæmopoetic tissue, they were administered in greater concentration than has hitherto been reported. It has been stated (Palmer, Cartwright and Wintrobe, 1952) that the administration of thyroid extract to rats maintained on a diet characterized by restricted caloric intake is followed by retarded rate of growth and leucopenia. Therefore, studies were also extended to observe the effect of chloramphenicol on thyroid-induced leucopenia.

MATERIALS AND METHODS.

Studies of the changes in the blood and the rate of growth of weanling rats were made in a series of six experiments (Table I). Chloramphenicol‡ [D(—) threo-2-dichloroacetamido-1-p-nitrophenyl-1, 3 propenidiol] and its 'diamine' derivative [D(—) threo-1-p-aminophenyl-2-amino-1, 3-propenidiol], hereafter referred to as 'diamine', were fed in concentrations of one, two, three, and five per cent of the diet. The control group of animals were maintained on normal basal diet *ad lib*. To rule out the effect of anorexia and other abdominal symptoms developing in rats on a diet containing chloramphenicol, in later studies, a second

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‡ Obtained by the courtesy of Parke, Davis and Company.

control group was pair-fed with the test group of animals. Each day the feed-cups were weighed and the amount of diet consumed by animals in the test group calculated. Their paired partners received the same amount of diet the next day. Rats were also maintained on a diet containing 0.6 per cent of desiccated thyroid* to which was added chloramphenicol (2 per cent of the diet).

TABLE I.

Experiments on toxic effect of chloramphenicol on weanling rats.

Experiment number.	Group.	Animals employed.	
1	1	10	Basal diet.
	2	10	Basal diet containing 1 per cent diamine.
2	1	12	Basal diet.
	2	12	Basal diet containing 3 per cent diamine.
	3	12	Basal diet containing 3 per cent chloramphenicol.
3	1	12	Basal diet.
	2	12	Basal diet containing 3 per cent chloramphenicol.
	3	12	Basal diet pair-fed with group 2.
4	1	36	Basal diet with 3 per cent chloramphenicol.
5	1	10	Basal diet with 5 per cent chloramphenicol.
	2	10	Basal diet pair-fed with group 2.
6	1	8	Basal diet with 0.6 per cent desiccated thyroid powder and 2 per cent chloramphenicol.
	2	8	Basal diet with 0.6 per cent desiccated thyroid powder.

*Only in experiment No. 4 were adult rats employed.

Diet.—A basal diet of the following percentage composition was used; casein (Sheffield, hot alcohol extracted), 20; sucrose, 64; lard, 11; salt mix†, 5; vitamins were added to this diet as follows (amount per kilogram of diet): thiamin hydrochloride, 12.5 mg.; riboflavin, 6 mg.; nicotinic acid, 60 mg.; pyridoxine hydrochloride, 10.0 mg.; calcium pantothenate, 25.0 mg.; inositol, 5.0 mg.; para-aminobenzoic acid, 5.0 mg.; pteroylglutamic acid, 15.0 mg.; choline chloride, 2.54 mg.; vitamin A, 23,000 units; vitamin D, 4,250 units; vitamin E, 7.6 mg.; vitamin K, 7.6 mg.

Animals.—Approximately 175 male, albino rats, of Sprague Dawley strain were used. The weanling rats ranged from 35 g. to 40 g. in weight, while the average weight of the adult rats was 250 g. They were housed in separate wire cages, and allowed water *ad libitum*.

*Thyroid extract—Abbot.

†The salt mixture was of the following percentage composition:

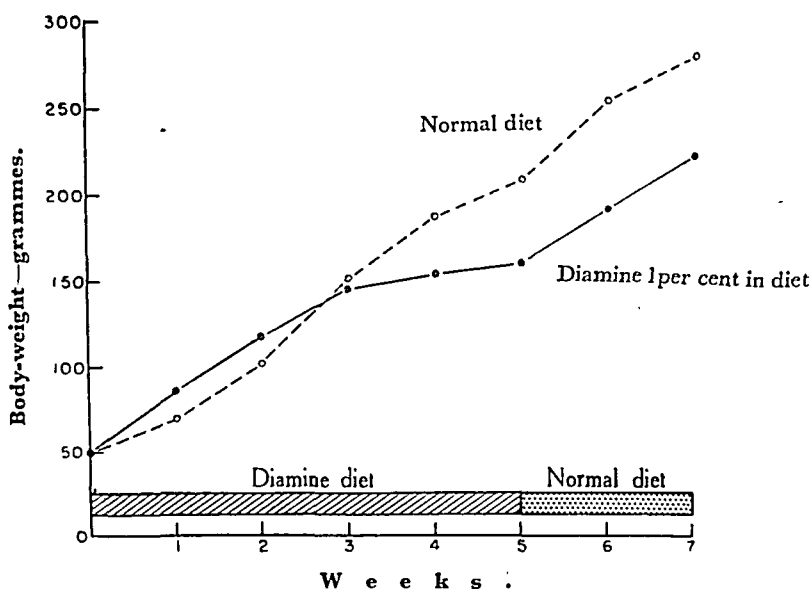
NaCl, 13.5; MgCO₃, 8.15; KH₂PO₄, 5.7; CaHPO₄, 46.3; KCl, 6.7; KI, 0.227; CaCO₃, 16.9; Fe₄(P₂O₇), 2.1; CuSO₄, 0.022; MnCl₂, 0.019; ZnO, 0.016; CoCO₃, 0.016.

Hæmatologic methods.—The Thomas pipette was employed for all white cell enumerations employing three per cent acetic acid as the diluting fluid, and a dilution of 1 in 20. Hæmoglobin determinations were carried out by the oxyhæmoglobin method using Evelyn photo-electric colorimeter. All hæmatologic determinations were made from free-flowing tail-vein blood at weekly intervals or more often if indicated.

RESULTS.

On a diet containing one per cent of 'diamine' a retardation in the growth of the animals was observed in the third week. On withdrawal of the drug from the diet in the fifth week, the rate of growth once again accelerated to equal the rate of growth of animals in the test group (Graph 1). No significant alterations were observed in the leucocyte count and the hæmoglobin content of the blood in these animals.

GRAPH 1.

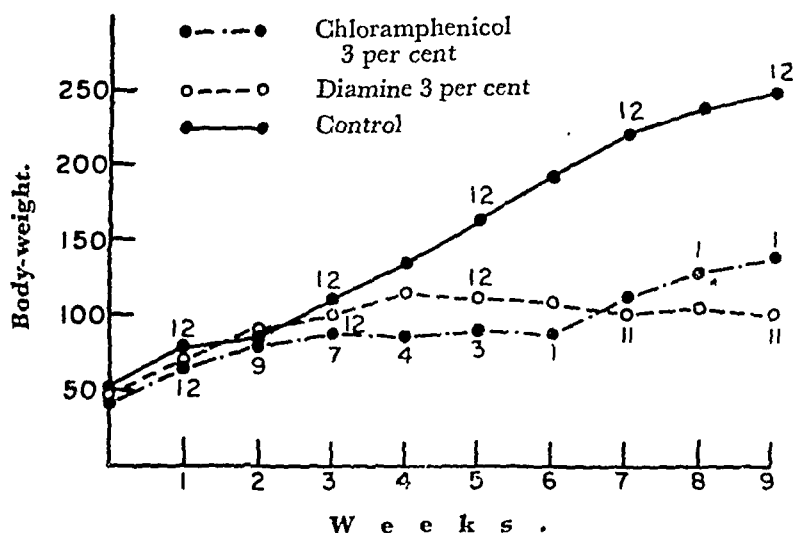


In another experiment, three groups of 12 animals each were used. The control group received normal basal diet *ad lib.* and each of the other two groups was kept on a diet containing three per cent 'diamine' and chloramphenicol, respectively. In the chloramphenicol-fed animals retardation in the rate of growth started in the second week. These animals developed severe toxic symptoms manifested by anorexia, abdominal distension, diarrhoea and vomiting. Their fur was poor, discoloured brown, and was scanty (Plate XI, figs. 1 and 2). In the 'diamine' group, no changes were observed other than previously described. The occurrence of death in the two groups was significantly different. It was

observed that by the end of six weeks, 90 per cent of the animals receiving chloramphenicol had died, whereas there was no death in the other two groups by that time (Graph 2). A reduction in the leucocyte count of chloramphenicol-fed animals was also observed as compared with those in the other two groups. In this group, the average leucocyte count was reduced from 17,000 per cu.mm. in the second week to 10,000 per cu.mm. in the fifth week (Graph 2a). There was no decrease in the level of haemoglobin in any of the groups.

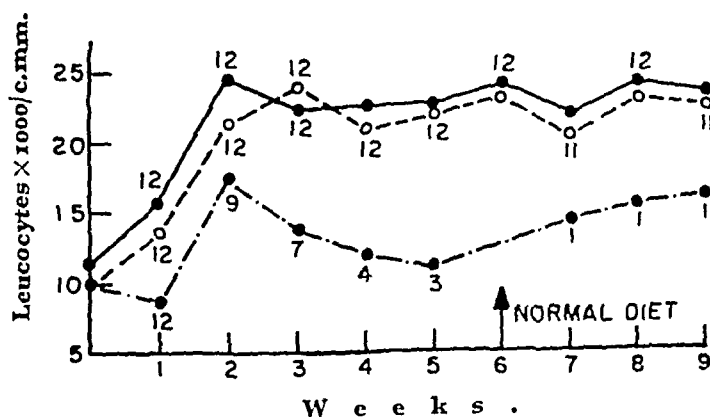
The significance of the difference in the total leucocyte count of the two groups could not be evaluated because of the occurrence of marked anorexia and diarrhoea in the test animals. However, when controlled with pair-feeding, there were no significant changes between the average leucocyte count of the two groups

GRAPH 2.



*Animals fed chloramphenicol and 'diamine' 3 per cent in diet.
Comparison of change in the body-weight and survival time.*

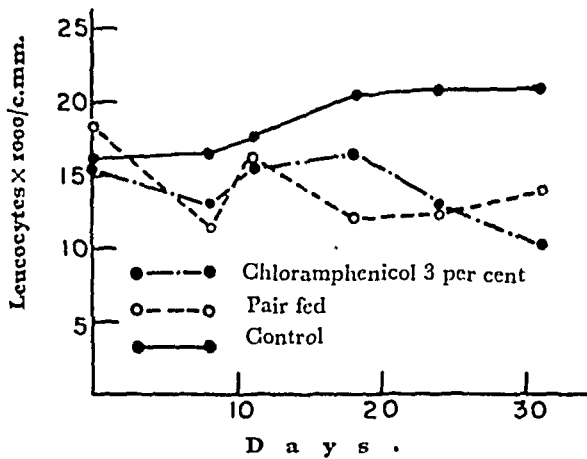
GRAPH 2a.



Comparison of change in the total leucocyte count.

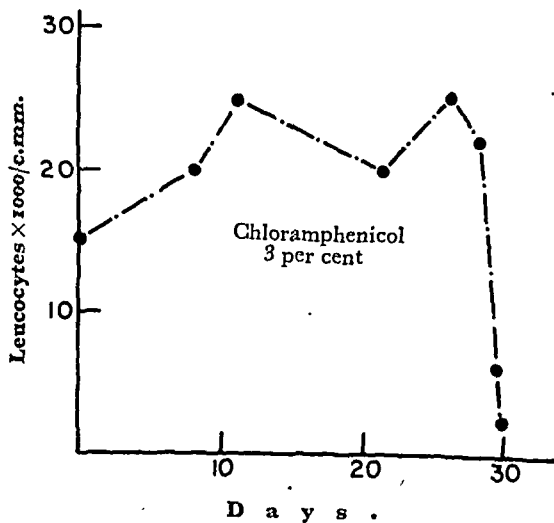
(Graph 3), although it was observed that a number of animals in the test group showed profound terminal leucopenia varying from 1,000 to 2,000 leucocytes per cu.mm. of blood (Graph 3a). The retardation in the rate of growth of the animals in the two groups was approximately the same (Graph 4), but the incidence of

GRAPH 3.



*Animals fed chloramphenicol 3 per cent in diet.
Changes in total leucocyte count as compared with pair-fed animals.*

GRAPH 3a.

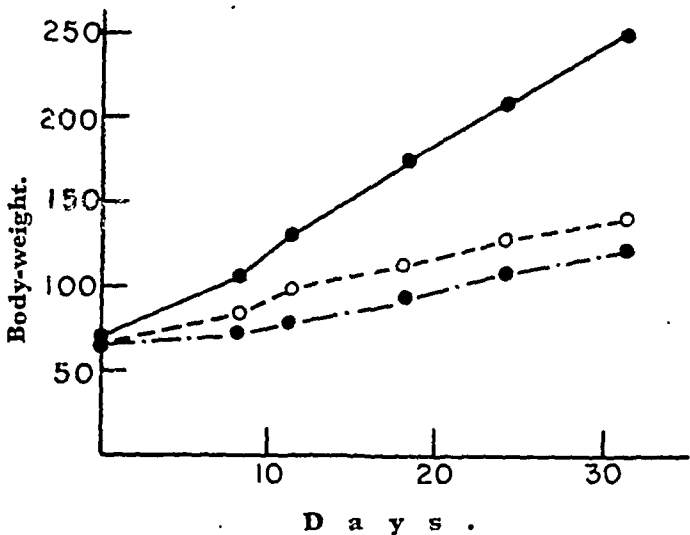


Terminal leucopenia in a rat.

death in the test group was again significantly higher than in the control animals (Graph 4a).

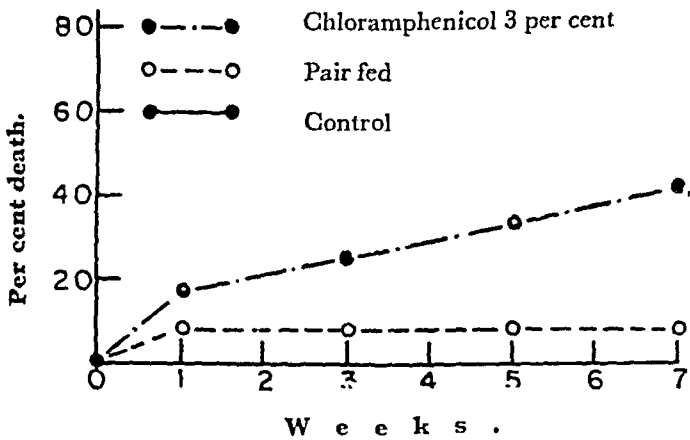
With an increase in the concentration of chloramphenicol to five per cent of the diet, the onset of symptoms was very rapid. The animals failed to grow, and within 1 to 2 days, became extremely malnourished, emaciated and died. The pair-fed partners lived almost twice as long and were distinctly in better shape. A rapid decrease in the leucocyte count of animals fed chloramphenicol was observed associated with profound weight loss. In these animals, the leucocyte count had fallen from 15,900 to 4,300/cu.mm. by the 3rd day, whereas pair-fed animals showed no appreciable change either in the leucocyte count or their weight

GRAPH 4.



Animals fed chloramphenicol 3 per cent in diet.
Changes in the rate of growth as compared with pair-fed and normal controls.

GRAPH 4a.



Comparison of rate of survival.

at this period (Table II). However, terminally leucocyte count was greatly reduced in pair-fed animals also.

In a study of the influence of chloramphenicol on the rate of growth and leucocyte counts of animals fed dessicated thyroid, no significant differences were observed between the test group and a control group receiving a similar but chloramphenicol free diet. Likewise adult rats fed chloramphenicol remained unaffected (Table III).

TABLE II.

Changes in total leucocyte count, body weight and survival of weanling rats fed chloramphenicol 5 per cent in diet.

Days.	ANIMALS SURVIVING:		WEIGHT G.:		LEUCOCYTES $\times 10^3$ PER CU.MM.	
	Pair-fed (10)	Capc. 5 per cent (10)	Pair-fed (10)	Capc. 5 per cent (10)	Pair-fed (10)	Capc. 5 per cent (10)
1	10	10	54.0	55.4	15.40	15.90
3	10	10	54.0	45.1	14.80	4.30
4	10	3	53.3	43.0	13.80	1.60
5	10	0	48.8	...	8.80	...
6	10	0	49.1	...	6.20	...
7	4	0	45.0	...	2.60	...
8	2	0	45.0	...	2.10	...

Capc. = chloramphenicol

TABLE III.

Changes in body weight and leucocyte count of adult rats fed chloramphenicol 3 per cent in diet.

Days.	Weight, g. (36)	Leucocytes $\times 10^3$ per cu.mm. (36).
1	264.0	18.70
2	256.0	18.40
3	255.0	19.80
5	250.0	18.21
8	242.0	17.40
15	264.0	20.32

DISCUSSION.

We have failed to observe any significant alterations in the blood picture of rats that could be attributed to the administration of chloramphenicol when it was given in concentration of 1 to 3 per cent of the diet. That, with an increase in the concentration of the drug to 5 per cent, the recipient animals developed profound leucopenia and weight loss, while it took twice as long for similar changes to appear in pair-fed partners shows that leucopenia was due to the administration

of the drug. However, it must be recognized that starvation may be a contributory factor. Keys (1950) has stated that starvation in human subjects may be accompanied by leucopenia. Whether the terminal leucopenia observed in partially starved pair-fed rats is similar in its causation to the above described leucopenia is a matter for speculation. Among a number of reports on the effect of chloramphenicol in the diet of various animals, significant anaemia was reported only in two dogs (Gruhzit *et al.*, *loc. cit.*). Recently, Rigdon *et al.* (1954) have produced anaemia in growing ducks on a diet containing chloramphenicol.

That the toxic signs other than death, herein described for weanling rats fed chloramphenicol, are due to the effect of the drug is clear from the observation that such signs failed to develop in the pair-fed control groups. With increasing concentration of the antibiotic in the diet, moreover, the onset of these symptoms was more rapid and more pronounced. It has been stated (Gruhzits *et al.*, *loc. cit.*) that oral administration of chloramphenicol to young rats leads to anorexia and weight loss. Bratton (personal communication) has also observed the occurrence of distention of abdomen and diarrhoea. It is reasonable to suppose that these manifestations are mainly due to an alteration in the bacterial flora of the alimentary tract under the influence of chloramphenicol. That the generalized poor state of these animals and the loss of fur is the direct effect of the drug, or due to some other mechanism has not been clearly ascertained. It is possible that chloramphenicol deprives the organism of some factor essential for its normal well-being, and that young animals are particularly susceptible to this mechanism, since we failed to observe similar changes in adult rats fed chloramphenicol in the amount of three per cent of the diet. In this respect the 'diamine' is relatively safe. This is also indicated by the relative influences these two substances have on the incidence of death in experimental animals.

The mechanism of the action of chloramphenicol, as an antibiotic or even as a toxic agent as herein reported, is not clearly understood. Recently, a number of reports have appeared on the role of this antibiotic as an anti-metabolite to certain essential aminoacids in bacterial systems. Wooley (1952) has shown that chloramphenicol acts as an anti-metabolite to phenylalanine for *E. coli*. A clear relationship to tyrosine has also been shown for chloramphenicol by Bergmann (1952). What relationship these observations may have to the abnormal hæmatologic reactions observed in certain individuals following the administration of this drug need further study. It may be that chloramphenicol may act to inhibit the 'utilization' of tryptophane and phenylalanine in these individuals. Further, in these individuals in whom 'sensitivity' to chloramphenicol has occurred, the drug may fail to be degraded in the usual fashion, and consequently may attain a sufficiently high concentration to inhibit activity of phenylalanine or tryptophane or both. Rigdon *et al.* (*loc. cit.*) state that the quantity of chloramphenicol necessary to produce anaemia in the duck is large as compared with that for man. It is significant in this respect that our animals developed leucopenia only when the concentration of the drug was raised to 5 per cent in the diet.

SUMMARY.

The influence of chloramphenicol and its 'diamine' congener upon the rate of growth and the blood of weanling rats was observed. The development of

anorexia, abdominal distension, coarse and discoloured hair, and diarrhoea appeared to be due to the action of the drug. The presence of this antibiotic in the diet of rats in amounts of one to three per cent of the diet did not have any demonstrable effect upon the body-weight or the average leucocyte count that could not be attributed to inanition alone. However, when administered as 5 per cent in diet, the recipient animals soon developed profound leucopenia while it took twice as long for similar change to appear in pair-fed controls. The incidence of death in animals fed chloramphenicol was found to be significantly higher than in those fed 'diamine' or pair-fed.

The author is grateful to Professor M. M. Wintrobe, M.D., Ph.D., and Dr. G. E. Cartwright, M.D., for suggesting this subject and their guidance. Gratitude is also extended to Dr. A. J. Samuels for close co-operation in this work.

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2. The rural population.

3. Small groups, with not less than 25 members in each, classed together on the basis of having the same occupation, religion or the same social status, such as businessmen, lawyers, doctors, Sikhs (on a high fat, non-vegetarian diet) and Marwaris (on a high fat, vegetarian diet). The results in these small groups are not yet complete. The present paper relates to the results obtained in the first two groups.

Industrial workers.—Four hundred and eighty-six labourers from the Delhi Cloth Mills were studied as the target was 5 per cent of the total industrial population of these mills which employ about 10,000 men working in rotation on 8-hour shifts. Although a full history was taken and a physical examination done on all of these 486 workers, it was possible to estimate the blood-lipids of only 197 men (i.e. 2 per cent of the total); this limitation being partly due to their reluctance to give blood and partly to the limited facilities available in our laboratory.

All the workers were men between 18 and 50 years of age. Their incomes ranged from Rs. 40/- to Rs. 120/- per month with an average of Rs. 60/-. They were all employed in mechanical occupations in the spinning, weaving, dyeing and printing departments of the mills and were classed as indulging in 'medium' activity (Keys *et al.*, 1956).

Rural population.—This was represented by persons attending the Primary Health Centre at Najafgarh which is 18 miles from Delhi. This Centre is fed by a population of about 23,000 from Najafgarh and its surrounding villages. It is proposed to examine 5 per cent of this population also. So far 566 persons, both men and women, have been studied. The number of blood samples analysed for serum lipids in this group was only 269, owing to the same limitations as in the case of industrial workers.

The income of this rural population was not accurately assessable as these men were engaged in rural occupations which did not bring them a fixed monthly income. Some of them were farmers, others labourers on the farms, a few were cobblers, carpenters, sweepers and petty landlords. The women were mostly housewives and helped on the farm. The only common factors were that they all lived in the rural environment of Najafgarh and the surrounding villages and came of a low income group.

METHODS OF INVESTIGATION.

Each individual was investigated under the following heads:—

1. History, including dietary history.
2. Physical examination.
3. Biochemical tests.
4. Electrocardiogram.
5. Screening of chest.

History.—All cases were interrogated on a special comprehensive pro forma printed for the purpose.

A complete dietary history with special regard to the fat and cholesterol content of the diet was taken by a process of interrogation. Food containers used in this part of the country were used to assess, as accurately as possible, the quantities of food eaten. The food values were later calculated from tables (Health Bulletin, 1951; and Okey, 1945).

Physical examination.—The criteria used in the diagnosis of atherosclerosis were as laid down by the New York Heart Association (1953).

(a) Coronary atherosclerosis was diagnosed on the following evidence:—

(1) History of myocardial infarction or of angina pectoris with or without electrocardiographic evidence.

(2) Purely electrocardiographic evidence of coronary artery disease, such as T-wave changes, a-v block, bundle-branch block, etc., when these changes were not due to any other disease. Such changes arising from other causes were naturally excluded from the study.

(3) Atherosclerosis of the aorta as shown by uncoiling and broadening of the aorta on fluoroscopy.

Other types of arteriosclerosis were also recorded in this survey in order to make the report comprehensive. They were:—

(4) Retinal arteriosclerosis as seen on fundoscopy.

(5) Arteriosclerosis of the muscular arteries, such as brachial and radial as shown by thickened and/or pulsating vessels.

(6) Cerebral arteriosclerosis as shown by a history or evidence of hemiplegia or other evidence of cerebro-vascular disease not due to any other obvious cause.

(b) *Hypertension.*—The criteria used for the diagnosis of hypertension were as laid down by the New York Heart Association (New York Heart Association, *loc. cit.*).

All the cases in this survey had a complete physical examination. In addition to recording the heights and weights, they were also classified according to body types.

Biochemical tests.—The serum lipids estimated were:—

(i) Total cholesterol [by the iron-reagent method of Zak *et al.* (1954)].

(ii) Phospholipids (estimated by applying the Fiske and Subbarow (1925) colour reaction on an alcohol-acetone extract of the serum).

The blood samples in all these cases were random specimens collected on calling the men from their work. In women, it was ascertained that they were neither pregnant nor menstruating at the time of collection of the blood sample.

In addition, a urinalysis for albumin and glucose was done in every case in order to exclude any case of undetected renal disease or diabetes.

Electrocardiography and fluoroscopy.—In the case of Delhi Cloth Mills workers it was possible to do electrocardiography in only 28 cases and fluoroscopy in 10 cases.

As Najafgarh was not electrified till recently it was not possible to do either electrocardiograms or screenings there except on a few cases who could come to Delhi. Since the installation of electricity in Najafgarh, 78 cases have had their electrocardiograms taken.

RESULTS OBTAINED.

The results in both these groups have been classified under two heads:—

- (i) Incidence of atherosclerosis and hypertension.
- (ii) Serum cholesterol levels and their variation with age, fat intake and cholesterol intake.

I. INDUSTRIAL WORKERS FROM THE DELHI CLOTH MILLS.

1. Incidence of atherosclerosis and hypertension (Table I).

TABLE I.

Incidence of hypertension and atherosclerosis among industrial workers from Delhi Cloth Mills and Najafgarh rural population.

Source.	Total number of case.	Hypertensives.	ATHEROSCLEROSIS:			Retinal atherosclerosis.	Thickened and pulsating peripheral arteries.
			History of coronary infarction or angina.	E.K.G. (positive.)	Aortic atherosclerosis.		
Delhi Cloth Mills, Industrial workers	486	6	<i>Nil</i>	<i>Nil</i> (28)*	4 (10)†	<i>Nil</i>	17
Najafgarh rural population, males	222	7	<i>Nil</i>	1 (34)*	4 (6)†	1	39
Najafgarh rural population, females	344	8	<i>Nil</i>	<i>Nil</i> (44)*	2 (4)†	<i>Nil</i>	14

* Number of cases in which E.K.G. was done.

† Number of cases in which fluoroscopy was done.

(a) *Atherosclerosis*.—The incidence of coronary atherosclerosis as shown by a history of angina pectoris or myocardial infarction was *nil* in this group. As electrocardiograms were taken of only 28 cases out of 486 men, i.e., 5.7 per cent of the total, this cannot be considered an adequate assessment. All the electrocardiograms were normal. Fluoroscopy for evidence of atherosclerosis of the aorta was possible only in 10 cases and was positive in 4 cases. Visible peripheral arterial pulsations showing evidence of arteriosclerosis of muscular arteries was found in 17 men, the youngest being 25 years old.

(b) *Hypertension*.—Out of 486 men examined, 6 were found to have hypertension (all of the benign essential type). All of them were between 30 and 40

years of age. The incidence of true hypertension in this group was thus 1.2 per cent.

2. Fat intake and serum cholesterol levels:

(a) *Dictary fat intake.*—The average daily fat intake in this group was very variable and ranged from 0 (i.e. people taking no animal fat and no vegetable oils at all) to 225 g. per day (Table III) with an average of 68 g. per day. Out of 197 men whose bloods were analysed, 126 (65 per cent of the total) had a daily intake of less than 75 g. Of these 197 men, 48 (25 per cent of the total) had a daily fat intake of less than 24 g., and 93 (47 per cent of the total) less than 49 g. Such large intakes as 200 g. or over of fat per day were found in the case of a few men who were taking 14 lb. of milk and 2 lb. of ghee (butter-oil) per week together with 1 to 2 lb. of meat also in some cases. It is the custom among some communities to drink ghee (butter-oil) by the cup as a source of strength. The source of fat in this group was always butter, milk or meat. No vegetable oils or hydrogenated fats were used for cooking as far as they knew.

For the 197 cases whose blood lipids were estimated, fats accounted for 24.5 per cent of total calories taken per day. Two specimen diets in this group showing the wide variation in fat intake are shown below:—

	I	II
Carbohydrates	533.8 g.	583.25 g.
Proteins	106.0 g.	101.90 g.
Fats	109.8 g.	14.05 g.
(a) Animal	104.2 g.	<i>Nil</i>
(b) From vegetable sources	5.6 g.	14.05 g.
Total calories per day	3,154	2,858
Percentage of calories derived from fats	31.3	4.4
	Per cent	Per cent
	(Animal fats	(Animal fats
	24.7 per cent)	<i>nil</i>)

(b) *Serum cholesterol levels.*—The average serum cholesterol levels with the range and mean in this group are given in Table II. They have been grouped as above and below 40 years because of the sharp rise with age reported in some studies after age 40 (Keys, Mickelsen, Miller, Hayes and Todd, 1950; and Keys *et al.*, 1952).

Variation in serum cholesterol levels with age.—As seen from Table II and the Graph there was a slight increase (16 mg. per cent) in the values for total cholesterol, between the ages of 20 and 50 years. The slight rise with age in the serum cholesterol was in the form of a steady plateau and the sharp rise after age 40, seen in studies elsewhere, e.g. Minnesota (Keys *et al.*, 1950) Massachusetts (Gertler *et al.*, 1950) was not noticed. The increase per year of life never exceeded 1 mg. in this series. The average rise per year of age for Minnesotans was reported as 2.2 mg. per cent. Statistically, the difference between the mean cholesterol levels of men aged 20 to 39 and 40 to 59 was not highly significant ($p=0.05$).

TABLE II.

Mean serum cholesterol levels in relation to age for industrial workers and rural population.

(With standard deviation and range.)

Age in years.	D.C.M. (MALES):		NAJAFGARH (MALES):		NAJAFGARH (FEMALES):		VARIATION IN TOTAL SERUM CHOLESTEROL LEVELS PER YEAR OF AGE FROM ONE DECADE TO THE NEXT:		
	Number of cases.	Total serum cholesterol in mg. per cent.	Number of cases.	Total serum cholesterol in mg. per cent.	Number of cases.	Total serum cholesterol in mg. per cent.	D.C.M. (Males).	Najafgarh (Males).	Najafgarh (Females).
10-19	6	169±30 (131-190)	23	170±40 (95-252)	23	167±40 (104-255)	...	...	...
20-29	87	164±25 (120-220)	49	186±42 (104-293)	56	180±47 (95-283)	-0.5	+1.6	+1.3
30-39	73	174±40 (106-265)	23	176±31 (129-297)	36	172±44 (113-252)	+1.0	-1.0	-0.8
40-49	20	180±40 (130-270)	21	191±21 (106-255)	13	180±39 (100-248)	+0.6	+1.5	+0.8
50-59	11	150±30 (112-155)	17	185±47 (115-297)	8	187±30 (140-242)	-3.0	-0.6	+0.7
Below 40 years	166	168 (106-265)	95	181 (95-297)	115	175 (95-283)			
Above 40 years	31	169 (112-270)	38	188 (106-297)	21	183 (100-248)			

Variation in serum cholesterol levels with fat intake (Table III and the Graph).—There was no great variation in serum cholesterol levels with increasing dietary fat intake. The highest values for total cholesterol were recorded with fat intakes of 200 g. daily, the level falling with a further increase in the intake of fat.

The differences in the serum cholesterol levels between the groups on varying fat intakes were not statistically significant. In view of the report of Keys *et al.* (*loc. cit.*) that the differences between populations in high and low fat intakes were most marked after 40 years of age, the men above 40 years of age were split into various fat intake groups. In these also no significant difference was found in the serum cholesterol levels, the mean cholesterol levels being 179.2 mg. per cent, 177.6 mg. per cent, 164.7 mg. per cent, and 166 mg. per cent for men above 40 years taking from 0 g. to 24 g., 25 g. to 49 g., 50 g. to 100 g. and 100 g. and over of fat per day, respectively.

Variation with cholesterol intake.

Table IV and the Graph showing the relation between the cholesterol intake and serum cholesterol levels, show no progressive increase in the serum total cholesterol with an increased cholesterol intake. The differences in the serum

cholesterol levels between the groups on varying cholesterol intakes were not significant. An analysis of variance done for the significance of the differences in the serum cholesterol levels of these men in various intake groups of fat and cholesterol showed that between sample variance was not more than within sample variance.

TABLE III.

Mean serum cholesterol levels in relation to daily fat intake for industrial workers and the rural population.

(With standard deviation and range of values.)

Fat intake g./day.	D.C.M. (MALES):		NAJAFGARH (MALES):		NAJAFGARH (FEMALES):	
	Number of cases.	Total serum cholesterol, mg. per cent.	Number of cases.	Total serum cholesterol, mg. per cent.	Number of cases.	Total serum cholesterol, mg. per cent.
0-24	48	162±38 (115-205)	33	171±40 (106-290)	43	176±42 (100-254)
25-49	45	170±40 (106-260)	30	182±44 (95-297)	41	174±40 (111-248)
50-74	33	163±40 (112-220)	24	178±35 (104-267)	14	173 (144-223)
75-99	26	168±38 (120-265)	15	172±38 (130-293)	12	165 (115-235)
100-124	12	170±41 (135-205)	5	171 (111-243)	3	173 (132-255)
125-149	12	160±38 (120-210)	10	212±41 (159-289)	...	...
150-174	8	168 (140-205)	4	202 (183-225)	...	...
175-199	6	188 (150-225)	5	172 (148-196)	...	...
200-224	4	155 (125-250)	3	220 (207-240)	...	...

II. NAJAFGARH POPULATION.

Five hundred and sixty-six cases (222 males and 344 females) were examined. The total number of blood samples analysed was 269.

I. INCIDENCE OF ATHEROSCLEROSIS AND HYPERTENSION.

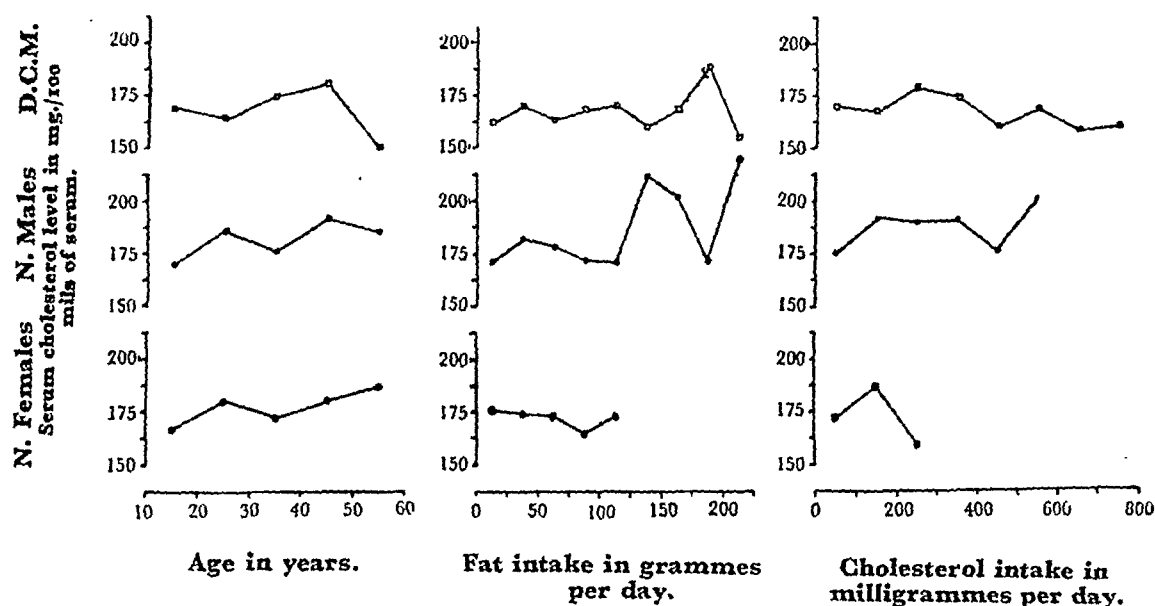
(a) Males—(Table I).

Atherosclerosis.—It was seen that the incidence of atherosclerosis as shown by a history of angina pectoris or myocardial infarction was *nil* in this group. Out of 34 electrocardiograms done only one showed evidence of coronary artery disease, an incidence of 3 per cent. And out of 6 cases whose chests were screened, 4 showed evidence of atherosclerosis of the aorta.

Retinoscopy was done in 10 cases and arterial changes were found only in one man. Arteriosclerosis of muscular arteries was found to be present in 39 cases of different ages, the youngest being 19 years of age.

GRAPH.

Variation in serum cholesterol levels with age, fat intake and cholesterol intake. For industrial workers (males) from Delhi Cloth Mills and Najafgarh rural population (males and females).



Hypertension.—This was present in 7 cases out of 222. There were 6 cases of benign essential hypertension and one of malignant hypertension. The incidence of hypertension was thus 3.2 per cent.

(b) *Females*—(Table I).

Atherosclerosis.—Out of 344 cases examined, there was not a single case of coronary artery disease as judged by a history of myocardial infarction or of angina pectoris. Electrocardiography was done on 44 cases and was normal in every case. Atherosclerosis of the aorta was present in 2 out of 4 females fluoroscoped. Both of them were above 40 years of age.

Hypertension.—Out of 344 cases examined, 8 had 'benign essential' hypertension. The incidence of hypertension in this group was 2.3 per cent.

The overall incidence of coronary artery disease in the rural group was not accurately assessable owing to the limitations mentioned but would appear to be very low. If only a history of angina or coronary thrombosis be taken as the criterion, the incidence may be regarded as *nil*.

2. FAT INTAKE AND SERUM CHOLESTEROL LEVELS.

Dietary fat and cholesterol intake: (Tables III and IV).—The daily intake of fat in this rural group ranged from 0 g. to 250 g. of fat with an average of 53.3 g.

(67 g. daily for males and 38.5 g. daily for females). The majority of subjects, however, i.e. 147 (54.7 per cent of the total) consumed less than 50 g. per day, and 76 (28 per cent of the total) took even less than 25 g. per day. The cholesterol intake ranged from 0 mg. to 640 mg. with an average of 132 mg. per day. The high intake of fat in some of these cases was accounted for as in industrial workers. Amongst this group fats contributed 24.1 per cent of the total caloric intake for males and 17.3 per cent for females. Nearly all these people were consuming only animal fats, such as butter, butter-oil and very few were using vegetable oils or hydrogenated fats.

TABLE IV.

Mean serum cholesterol levels in relation to daily cholesterol intake for industrial workers and the Najafgarh rural population.

(With standard deviation and range of values.)

Cholesterol intake mg./day.	D.C.M. (MALES):		NAJAFGARH (MALES):		NAJAFGARH (FEMALES):	
	Number of cases.	Total serum cholesterol, mg. per cent.	Number of cases.	Total serum cholesterol, mg. per cent.	Number of cases.	Total serum cholesterol, mg. per cent.
0-99	64	170 $\pm$ 35 (114-293)	52	175 $\pm$ 45 (95-290)	80	172 $\pm$ 42 (100-283)
100-199	50	168 $\pm$ 38 (105-249)	31	192 $\pm$ 48 (104-297)	38	187 $\pm$ 48 (104-275)
200-299	42	180 $\pm$ 42 (112-265)	20	190 $\pm$ 43 (129-293)	15	159 $\pm$ 30 (115-235)
300-399	12	175 $\pm$ 39 (125-210)	12	191 (111-252)	...	...
400-499	16	162 $\pm$ 35 (120-215)	8	177 (138-216)	...	...
500-599	5	170 $\pm$ 42 (150-235)	5	201 (148-255)	...	...
600-699	4	160 $\pm$ 48 (140-230)	...	...	...	...
700-799	1	162	...	...	...	...

Serum cholesterol levels.—The total cholesterol levels were slightly higher than those found in the industrial group, both above and below 40 years of age (Table II).

Variation with age: (Table II and the Graph).—As seen from the Graph and Table II there was an increase in the values for total serum cholesterol by 15 and 20 mg. per cent between the age groups of 10 to 19 and 50 to 59 years, among men and women, respectively. The rise, however, was gradual and there was no sharp ascent after age 35 or 40 as noted in studies elsewhere. Although the rise was sharpest between the ages of 30 and 50, it was not more than 20 mg., and the sharpest rise per year (1.6 mg. cholesterol) occurred between 20 and 29 years. The rise would appear to be in the shape of a gradual plateau.

For both men and women, the differences in the mean serum cholesterol levels in different decades were not statistically significant.

Variation with fat intake: (Table III and the Graph).—There was undoubtedly an increase in the total cholesterol level with increase of fat intake, but this rise could by no means be called a steady one. The highest serum cholesterol recorded was certainly in those with the largest fat intake among the men. This was not true for women, however, who lived on relatively less fat than the men.

The differences in the mean serum cholesterol levels of both men and women in various fat intake groups were not statistically significant. Even after taking men and women above 40 years of age and splitting them in various fat intake groups, their mean serum cholesterol levels did not differ significantly.

Variation with cholesterol intake: (Table IV, and the Graph).—Here again, the highest cholesterol levels recorded in the men were in those with the highest daily intake of cholesterol but in women the level was higher for the group taking 100 mg. to 199 mg. of cholesterol per day than for the group taking 200 mg. to 299 mg. of cholesterol per day. However, there was no obvious correlation between the cholesterol intake and the serum-cholesterol levels. The differences in the mean serum cholesterol levels of both men and women in various cholesterol intake groups were not statistically significant.

An analysis of variance done for the significance of the differences in the serum cholesterol levels of these men and women in various decades of age and different intake groups of fat and cholesterol showed that between sample variance was not more than within sample variance.

Body weight.—The weight in pounds per inch of height was calculated so as to give an index of relative obesity of the individual. Almost all the cases studied in this series had a relative weight lower than the ideal of 2.1 lb. to 2.4 lb. for men and 1.9 lb. to 2.1 lb. for women (Metropolitan Life Insurance Company, Statistical Bureau). The average relative weight for industrial workers was 1.8 lb. for males from Najafgarh 1.8 lb., and for females from Najafgarh 1.7 lb.

DISCUSSION.

Incidence of atherosclerosis.—There has not been any survey of heart disease among industrial and rural groups in India as far as can be ascertained from the literature.

The only statistics obtainable are for urban groups, mostly from hospital patients. The incidence of atherosclerosis and hypertension according to various workers varied from state to state. In Delhi hypertension accounted for 21.1 per cent and coronary atherosclerosis for 11.8 per cent of all heart cases in a five year survey (Padmavati, 1957). The figures are higher for both these diseases in certain other parts of India (Malhotra, 1951; and Vakil, 1954). Although the best index of the incidence of atherosclerosis in any population would be a post-mortem survey for incidence of all grades of coronary atherosclerosis, it is a very difficult ideal to achieve in India where autopsies are almost unobtainable. A history of angina pectoris or coronary thrombosis, preferably with electrocardiographic evidence would seem to be the best clinical criterion. Studies elsewhere have suggested that aortic and coronary atherosclerosis are not always correlated and, therefore, the incidence of aortic atherosclerosis would not be perhaps such a good guide (Keys and White, *loc. cit.*).

Among the 486 industrial workers, all males, and 566 members of both sexes of the rural group, the incidence of coronary atherosclerosis was practically *nil*, judging from the history and electrocardiograms, the latter possible in only 10 per cent of cases. Of the two groups, only the rural group showed a single positive electrocardiogram out of 78 taken, an incidence of 1.3 per cent.

Comparable figures from other countries for coronary artery disease are also lacking for selected population groups as those under discussion here. The figures available in other countries are either post-mortem statistics showing the incidence of various degrees of coronary atherosclerosis in a large number of post mortems or are hospital and private patient figures. Table VI represents the incidence of coronary artery disease among all medical admissions in various countries and it is noted that the incidence for Delhi is very low indeed, being the same as for Naples, the lowest on the list. This is in keeping with the data obtained in the present survey pointing to a low incidence of coronary atherosclerosis. Compared to figures for Western Europe the incidence is anything from 8 to 15 times less frequent. Figures from the so-called backward countries are more comparable. Higginson and Pepler (1954) in an autopsy study on 934 Bantu, found only one case of coronary occlusion and state that incidence of grade III coronary atherosclerosis is barely a fifth of that found among Americans of equal age. In Japan, Kimura (Quoted by Keys and White, *loc. cit.*) in a study of 10,000 autopsies found only 75 cases of myocardial infarction and concluded that the incidence of severe coronary atherosclerosis was about one-tenth that in persons of the same age in the United States. In Okinawa the rarity of coronary artery disease at autopsy among Okinawans has also been noted (Benjamin, 1946). Although some publications in India for patients in the high socio-economic groups seen in private practice would indicate that coronary atherosclerosis was much higher than in the poor socio-economic group (Gopalan and Ramanathan, 1956; and Malhotra, 1951) and in our own observations, the incidence among a group of professional men studied was 10 per cent (unpublished observations), these figures are nowhere near those of Western Europe or the United States.

† *Incidence of hypertension.*—In this study, in contrast to the figures for atherosclerosis the figures for hypertension (not the same as hypertensive heart disease) are accurate. Among 566 members of the rural groups of both sexes the incidence was 2.7 per cent and in 486 of the industrial group the incidence was 1.2 per cent. The figure for hospital statistics for Delhi out of all medical admissions was 3.2 per cent (Padmavati, 1958). Out of all heart cases hypertension accounted for 21.1 per cent of cases. Compared to other countries (White, 1951) the incidence would appear to be low although the figures are not actually comparable because the statistics relate here to particular population groups.

Dietary fat intakes.—With regard to dietary fat intake those given in Table V are all post-war statistics for different parts of India as other surveys available in the literature are more than 15 to 20 years old. It will be seen that the fat intake varies in different parts of India and according to the economic status of the population. Thus, in Coonoor among the low economic group Gopalan and Ramanathan (*loc. cit.*) have reported a daily consumption of 10 g. to 30 g. of fat and among the high income group of 85 g. to 120 g. In a Bombay rural survey (Diet and Nutrition Studies in Rural Areas, 1956) the figures for dietary fat intake

varied from 27.1 g. to 73.7 g. per day. Although the figures for Delhi are higher than for other parts of India, it will be evident from Table III that 50 per cent of the total population consumed less than 50 g. of fat per day. Fats accounted for 24.5 and 24.1 per cent of total calories in the industrial and rural groups, respectively.

TABLE V.

Dietary fat intake in different parts of India in g. per day.

(Range in brackets.)

DELHI:		BOMBAY RURAL POPULATION:			COONOR:		
Industrial workers.	Rural population.	High income.	Medium income.	Low income.	Average.	High income.	Low income.
68 (0-225)	53.3 (0-250)	73.7	52	27.1 —	47.6	(85-120)	(10-30)

TABLE VI.

Incidence of coronary artery disease out of all medical cases from 40 to 70 years of age.

(Hospital figures.)

			Per cent.
I. Italy (Keys and White, <i>loc. cit.</i>)	...	Naples	1.7 to 2.0
		Bologna	5.3
II. Sweden (Keys and White, <i>loc. cit.</i>)	...	Lund	14.0
III. U.S.A. (Keys and White, <i>loc. cit.</i>)	...	Boston	18.2
		Twin cities	26.2
IV. India (Padmavati, <i>loc. cit.</i>)	...	Delhi	1.7

A comparison with other countries would suggest that the fat intake of the Delhi population (low socio-economic groups) was similar to that of Cape Coloreds, and of men from Sardinia, Bologna (Keys *et al.*, 1956), and Spain (Keys *et al.*, 1954). It was higher than the figures reported for Nigerians (Mann *et al.*, 1955), Bantus (Bronte Stewart *et al.*, 1955) and Naples firemen (Keys *et al.*, 1956). It was far below the figures for Cape Europeans (Bronte Stewart *et al.*, *loc. cit.*), Swedes (Keys *et al.*, 1956), men in Great Britain (Keys and Keys, 1954) and of course those in the United States (Keys *et al.*, 1950). The age groups are mostly comparable.

Serum cholesterol levels.—Very few comparable data exist from other parts of India regarding serum cholesterol levels also. Early references from Calcutta (Boyd and Roy, 1926; Bose and De, 1936) and Lucknow (Ghose, 1933) were low compared to the present series (Table VII). However, the methods used were very different from present day ones. The figures from Coonoor for low income groups were lower than for Delhi and the figures for high income groups were more

comparable. The figures for Defence personnel in Delhi (Nath *et al.*, 1957) were somewhat higher. However, these men belonged to a high socio-economic group.

TABLE VII.

Serum cholesterol levels in Indian males (normals).

Place.	Calcutta (Boyd and Rey, <i>loc. cit.</i>)	Lucknow (Ghose, <i>loc. cit.</i>)	Calcutta (Bose and De, 1936.)	Coonoor (Gopalan and Rama- nathan, <i>loc. cit.</i>)		Delhi (Nath, Gopalan and Iyer, <i>loc. cit.</i>)	Delhi.	
				Low income	High income	Defence personnel	Industrial workers.	Rural population.
Method used	Myers Wardell method.	Bloor's method.	Myers Wardell method.	Abel and Kendall's method.		Sperry and Webb method.	Iron reagent method of Zak <i>et al.</i>	
Total serum cholesterol in mg. per cent.	82 to 184	110 to 180	120 to 160 (mean 140)	121 to 126	132 to 170	177 to 215	168·7	179·5

The cholesterol levels in Delhi were slightly higher in the rural population than in the industrial workers. Compared to the other countries the cholesterol levels in the industrial workers were similar to those of the Bantus (Bronte Stewart *et al.*, *loc. cit.*) and Naples firemen (Keys *et al.*, 1956), both of whom lived on lower fat diets. The cholesterol levels in the rural population were slightly lower than those of Cape Coloreds (Bronte Stewart *et al.*, *loc. cit.*) and much lower than for poor Spaniards (Keys *et al.*, 1954) and similar to those of Bolognese (Keys *et al.*, 1956), Sardinians (Keys *et al.*, 1956) and the patient controls from Edinburgh (Oliver and Boyd, 1953). The Cape Coloreds, Bologna policemen and the Sardinians lived on approximately the same fat intake as the Delhi groups. The levels were much lower than those for Western Europe (Keys and Keys, 1954; Keys *et al.*, 1954; and Oliver and Boyd, *loc. cit.*) and the U.S.A. (Gertler, Garn and Bland, 1950; and Keys *et al.*, 1950) and significantly higher than those of Nigerians (Mann, Nicol and Stare, *loc. cit.*).

FACTORS INFLUENCING THE SERUM CHOLESTEROL LEVELS.

Age.—In the group of industrial workers the rise in serum cholesterol levels from the decades 10 to 29, and 30 to 49 was probably significant, the value of 'P' being 0·05. In the Najafgarh group there was no significant increase with age either in males or females. The increase per year of life did not exceed 1·6 mg. per cent at any time in both the industrial and rural groups and as stated before the rise was in the form of a steady plateau giving a total difference of only 16 mg.

and 20 mg. per cent, respectively, in the value of serum total cholesterol between the ages of 20 and 50 years.

The sharp rise noted after 40 years seen in studies elsewhere, such as in Minnesota (Keys *et al.*, 1950) and Massachusetts (Gertler *et al.*, 1950) was not noticed in this study. The average rise per year of life in the Minnesota studies was 2.2 mg. Our studies would seem to resemble those of Oliver and Boyd (*loc. cit.*) at Edinburgh, and of Keys *et al.* in Neapolitans, the Bantus and the poor Spaniards (Keys *et al.*, 1954).

In this study even after the age of 40 years there was no significant increase in the serum cholesterol levels of these subjects with an increasing fat intake. This would support the contention of Keys *et al.* (1952) that the rise of serum cholesterol with age is most marked in populations on high fat diets.

Fat intake.—We have not been able to establish any significant increase in the serum cholesterol levels with increasing fat intake in any of the groups studied. It must, however, be remembered that most of the subjects in all these groups were on a diet of fat content below 50 g. per day and the number on a high fat intake was small. It is interesting to note that in the small group of businessmen, and professional men we have studied so far (unpublished data) the blood cholesterol levels and the fat intake were the highest recorded in our whole series. Professional men (doctors) have an average blood cholesterol level of 264.5 mg. per cent. Further studies on these high socio-economic groups which are being completed will doubtless throw further light on this aspect of the problem. That a rise in serum cholesterol occurs on a high fat diet has been proved in feeding experiments on humans (Keys, 1952; Ahrens *et al.*, 1955; Hildrith *et al.*, 1951; Kempner, problem 1948; Keys *et al.*, 1950; and Chapman, *loc. cit.*)

Further, epidemiological studies throughout the world would suggest that with increasing dietary fat intake the serum cholesterol levels also rise.

Cholesterol intake.—We have not been able to demonstrate any significant rise in the serum cholesterol level with increasing cholesterol intake. This would appear to be in keeping with the findings of others (Keys and Keys, 1954; Keys *et al.*, 1950; and Wilkinson, Blecha and Reimer, 1950).

Physical activity.—Most of the individuals in both these groups fell into the range of 'medium' activity (Keys *et al.*, 1956). It was, therefore, not possible in these two groups on similar fat intakes to study the effect of physical exertion on the serum cholesterol levels.

SUMMARY AND CONCLUSIONS.

1. In a survey of 566 rural people and 486 industrial workers in Delhi, the incidence of hypertension was found to be 1.2 per cent in industrial workers and 2.6 per cent in the rural population; that of coronary artery disease was *nil* as judged from the history and about 1.3 per cent in Najafgarh if the small number of electrocardiograms taken were considered.

2. The average fat intake and serum cholesterol levels for the two groups were as follows:—

Group.	Fat intake in g. per day.	Per cent of calories from fats.	TOTAL CHOLESTEROL IN MG. PER CENT	
			Below 40 years.	Above 40 years.
Industrial workers	68	24.5	168.0	169.0
Najafgarh (males)	67	24.1	180.3	192.4
Najafgarh (females)	38.5	17.3	174.0	182.4

3. There was no correlation between the serum cholesterol levels and the dietary fat intake or the dietary cholesterol intake.

4. The serum cholesterol level tended to increase with age but the rise was very gradual and did not show the sharp upward trend noted in some studies elsewhere.

5. The figures obtained at Delhi for dietary fat and for blood cholesterol levels were compared with those for other parts of India from the few data available and the differences discussed.

6. The figures for Delhi were compared for both fat intake and cholesterol levels with figures from other countries. It was found that both as regards fat intake and the total cholesterol levels, Delhi resembled the results of survey among the Bantu, Cape Coloreds, Neapolitans, and Bolognese in Italy. The significance of various factors considered responsible is discussed.

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EXPERIMENTAL PEPTIC ULCERATION.

Part II.

THE SIGNIFICANCE OF PEPTIC ACTIVITY.

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SCHIFFRIN and WARREN (1942) showed that an important factor in the production of peptic ulceration was pepsin of the gastric juice; free acidity alone did not cause marked ulceration, but acid pepsin solution, when introduced into the stomach, produced ulceration and even perforation. Babkin (1950) noted that by inactivating pepsin without changing the acidity, the life of dogs with experimental peptic ulceration could be prolonged. LeVeen (1947) observed that pepsin was essential for the production of ulceration and suggested that the role of acid was only to enhance the peptic activity. Mann (1935), on the other hand, produced experimental gastric ulceration by continuous drip of hydrochloric acid in the stomach. Winkelstein and Berg (1938) showed that hyperchlorhydria was an essential factor in the causation of peptic ulceration. Grossman (1947) also demonstrated that acid alone could damage the gastric mucosa. Zaidi and Mukerji (1958) in a previous communication showed that high degree of ulceration was associated with higher values of free acidity.

Babkin (*loc. cit.*) suggested the necessity for further investigations in the relationship of pepsin to experimental peptic ulceration. Grossman (*loc. cit.*) was of opinion that more proof was needed before acid could be rejected as the main factor in the causation of peptic ulceration. An attempt has, therefore, been made to study these aspects and also the relationship of peptic activity and pepsinogen content of gastric tissue to experimental peptic ulceration.

Experimental Peptic Ulceration.

MATERIALS AND METHODS.

Animals.—Male albino rats of the Institute strain, average weight 220 g., were used.

Production of peptic ulceration.—The technique described by Shay (1945) with suitable modifications was used. The rats were fasted for 48 hours and water was allowed *ad lib.* throughout the starvation period. After this period the animals were anæsthetized with ether, abdomen opened and pylorus ligated avoiding nearby vessels and nerves. The animals were sacrificed with ether anæsthesia sixteen hours after operation.

Protection of peptic ulceration.—Protection from ulceration of the animals was brought about by injecting anticholinergic drug, oxyphenonium bromide, in multiple doses. First injection of 0.5 mg./c.c. was administered intraperitoneally 15 minutes before the ligation and second and third injections of the same dose three and six hours respectively after the first injection. With this multiple dose schedule an effective prevention from ulceration was made possible.

Histopathological technique.—Autopsy was performed on all the animals sacrificed. After opening the abdominal cavity the stomach was inspected *in situ* for distension, congestion and perforation. It was then taken out, a small slit made in the duodenum along the greater curvature and the contents drained in a beaker. The stomach was then opened and inspected for any ulceration. The tissue blocks from stomach were fixed in 10 per cent formol saline and Regaud's fluid. After complete fixation the blocks were embedded in paraffin and sections cut at 5 μ . Sections were stained with hæmatoxylin and eosin and for pepsinogen by Bensley's neutral gentian stain (Cowdry, 1948).

BIOCHEMICAL TECHNIQUE.

Pepsinogen.—All the stomachs, except those used for histological purposes, were washed in cold water to remove food particles and homogenized individually in 100 c.c. of distilled water for five minutes in a Waring blender. The pepsinogen content of the aliquots after suitable dilution with 0.001 N-HCl, was determined by the method of Hirschowitz (1953). Instead of hæmoglobin, casein substrate was used. Results have been expressed as units of pepsin per stomach.

Peptic activity.—The gastric contents from each animal were centrifuged to remove any food particles. Peptic activity of gastric juice was estimated as described by Glick (1955) using phenol reagent and casein substrate. The results have been expressed as 'pepsin unit'.

Free and total acidity.—Free and total acidity was titrated with 0.01 N-NaOH using Topfer's reagent and phenolphthalein as indicators and expressed as m.eq/l.

PLAN OF THE EXPERIMENT.

The animals were divided into three groups. Group I of 10 animals fasted for 48 hours was kept as normal control. Group II consisted of 20 animals in which ulceration was produced. Group III included 15 animals which were ligated



FIG. 1. Left. Normal stomach. Right. Ulcerated stomach.



FIG. 2. Normal stomach. Peptic cells contain dark staining pepsinogen granules (Bensley's neutral gentian stain $\times 350$).



FIG. 3. Ulcerated stomach. Pepsinogen granules have disappeared from most of the peptic cells (Bensley's neutral gentian stain $\times 350$).

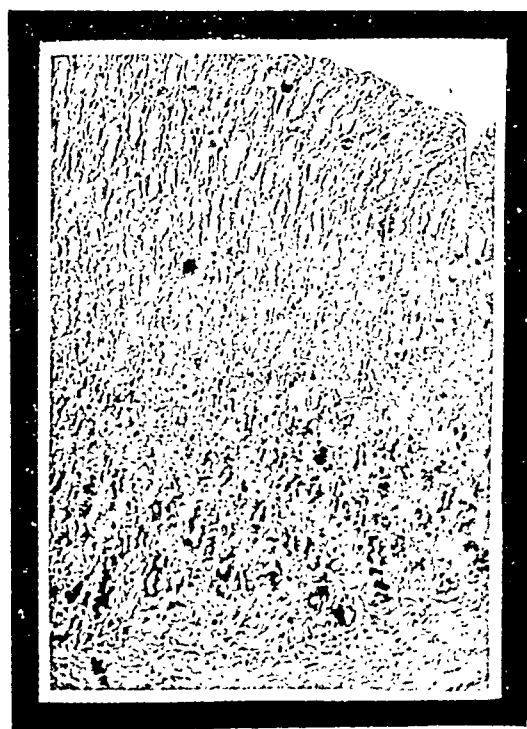


FIG. 4. Stomach protected from ulceration. Peptic cells contain dark staining pepsinogen granules (Bensley's neutral gentian stain $\times 350$).

group II received 0.5 g. of sodium bromide. This procedure was continued for 48 hours after the last dose of lactose. Four rats in each of the groups I, II and III (controls), were kept separately for the examination.

RESULTS

(a) Histopathology:

Gross appearance.—The naked eye examination of stomach *in situ* of groups I and III showed normal appearance. The mucosa was greyish pink and had no ulceration. The stomach in group II showed marked distension, congestion of the stomach and ulceration in seventeen animals. Ulcers were confined only to the cardiac portion of the stomach, their severity and extent varied from superficial to deep ulceration and even perforation (Plate XII, fig. 1).

The gastric contents of the ulcerated animals were chocolate brown in colour.

Microscopic appearance.—Animals of groups I and III showed normal appearance of the stomach. There was no evidence of congestion, inflammation or ulceration. In group II, where ulceration was produced, marked congestion of the blood vessels and diffuse infiltration with acute inflammatory cells mostly polymorphs was seen in almost all the layers of stomach. Ulceration varied from superficial hemorrhage to deep damage of the muscular and serous layers. The perforation showed excited oedematous margins with marked infiltration of acute inflammatory cells.

Pepsinogen granules stained with Bensley's neutral gentian stain, in the normal (control) and protected group of animals filled the peptic cells (Plate XII, figs. 2 and 4). In ulcerated group, however, the pepsinogen granules had disappeared from most of the peptic cells (Plate XII, fig. 3). The peptic cells were shrunken and the lumen of the glands had become wider as compared to the normal and the group protected from ulceration.

(b) Biochemical:

Pepsinogen of the stomach.—The pepsinogen contents of the stomach in the normal (control) and protected group of animals did not differ at 5 per cent level of significance. The pepsinogen in ulcerated group was significantly lower than the normal animals at 5 per cent level and with the protected group at 1 per cent level (see Table I and the Graph).

Gastric juice.—The volume of the gastric juice obtained after 48 hours fasting in the normal animals had a mean of 0.2 c.c. The volume in the protected and ulcerated group had a mean of 2.0 c.c. and 2.6 c.c. respectively, the difference between the two was not statistically significant at 5 per cent level.

Peptic activity.—The results of peptic activity estimations of the gastric juice are shown in Table I and the Graph. The mean values of peptic activity in the ulcerated group are higher than the normal and significantly higher than protected group at 5 per cent level.

Pepsinogen-peptic activity.—The relationship of pepsinogen and peptic activity is apparently the same in the normal and protected animals as evident from the pepsinogen-peptic activity ratio. In ulcerated group, however, the pepsinogen peptic activity ratio is significantly lower than the protected and normal groups. The mean values of pepsinogen and peptic activity are summarized in Table I and the Graph :—

TABLE I.

Gastric tissue pepsinogen and peptic activity of the gastric contents.

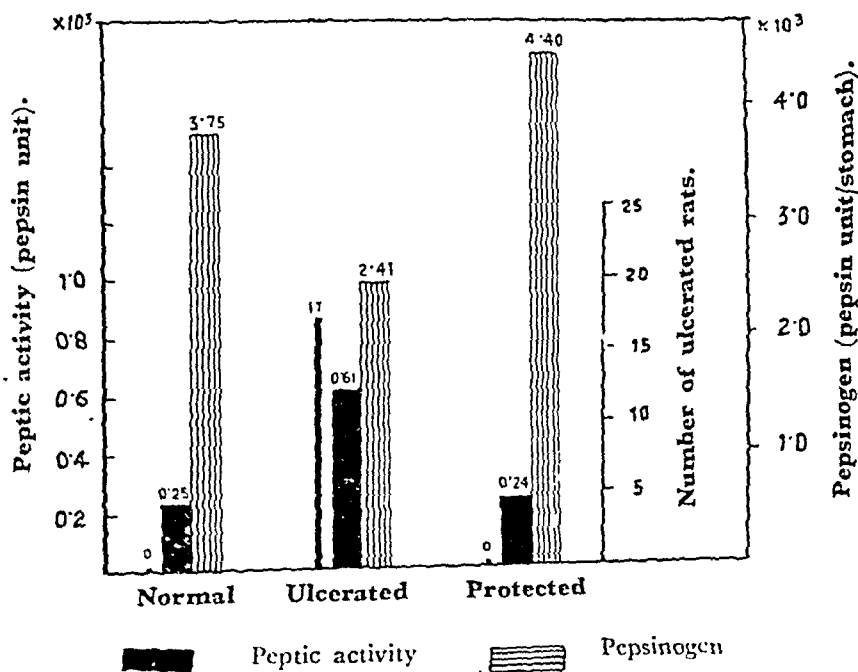
Group.	Number of animals.	PEPTIC ACTIVITY:	PEPSINOGEN:	Pepsinogen/peptic activity ratio.
		P.U. $\times 10^3$.	P.U./stomach $\times 10^3$	
I (Normal) ...	10	0.25*	3.75 ± 0.42	15.00
II (Ulcerated) ...	20	0.61 ± 0.08	2.41 ± 0.30	3.95
III (Protected) ...	15	0.24 ± 0.12	4.40 ± 0.35	18.33

Peptic activity—('t' test) The mean value for group II is significantly higher than for group III at 5 per cent level ($P < 0.05$).

* This group shows a variance significantly lower than in other groups and has not been used for comparison.

Pepsinogen—('t' test) The mean value for group II is significantly lower than for group III at 1 per cent level ($P < 0.01$) and group I at 5 per cent level ($P < 0.05$), groups I and III do not differ at 5 per cent level of significance.

GRAPH.



— Ulcerated rats.

Mean value of pepsinogen and peptic activity.

Acidity.—The mean values and the range of free and total acidity is shown in Table II. In the normal animals the mean value of free acidity was 4 m.eq/l. and in protected animals 6 m.eq/l., respectively. In the ulcerated group the mean value of free acidity was 51 m.eq/l., eight animals out of twenty showed free acidity less than 32 m.eq/l. and the values above 32 m.eq/l. ranged up to 100 m.eq/l. with the exception of one animal which had a very high value of 204 m.eq/l.

TABLE II.

Summary of results of acidity of gastric contents (groups I, II and III.)

Group.		I (normal).	II (ulcerated).	III (protected).
Number of animals :		10	20	15
Volume c.c.	Mean ...	0.2	2.6	2.0
	Range ...	0.1-0.3	1.1-5.7	1.1-3.7
Free acidity m.eq/l.	Mean ...	4	51	6
	Range ...	0-40	0-100	0-48
Total acidity m.eq/l.	Mean ...	14	98	59
	Range ...	10-20	30-134	26-110

(c) *Comparative study of histochemical and biochemical findings of pepsinogen in the gastric tissue.*—The histological study of pepsinogen and the chemical estimation of pepsinogen in the gastric tissue showed a direct relationship. In the normal and protected group peptic cells were filled with pepsinogen granules and greater amount of pepsinogen was estimated in the gastric tissue. In the ulcerated group fewer pepsinogen granules were seen in the section and lower pepsinogen values in the whole gastric tissue were obtained.

DISCUSSION.

In the present experiments, acute peptic ulceration was produced after the ligation of pylorus within a period of 16 hours. The site of ulceration was confined to the cardiac portion of the stomach and the severity of ulceration varied from mucosal hæmorrhage to deep ulceration. These findings are in general agreement with those of Barrett *et al.* (1953), though our experiments did not produce ulceration as consistently as reported by them. Our experimental results as regards protection against ulceration with oxyphenonium bromide show that in multiple doses there is complete prevention of its occurrence which may be due to anticholinergic effect of the drug.

Bowie (1940) noted, in cats, maximum number of peptic cells near the middle of the greater curvature, but those diminished in number towards the pylorus and cardiac end. Zaidi and Mukerji (*loc. cit.*) also showed that in histamine-induced ulceration in guinea-pigs the degree of ulceration in the fundus was several times higher than in the pylorus. In our experiments, the ulceration produced by the ligation of pylorus was confined to the cardiac portion devoid of peptic cells. The site of ulceration, therefore, is not related to the distribution of peptic cells.

Hirschowitz *et al.* (1956) noted that pepsinogen contents of rat's stomach were decreased after adrenalectomy and Scudder *et al.* (1938) observed that changes

produced through obstruction of gastro-intestinal tract were markedly similar to those seen in adrenal insufficiency. In the present experiments in ulcerated and ulcer-prevented group of rats, the conditions of obstruction were identical and if adrenal insufficiency played a major part, it should have manifested itself equally in both the groups. The pepsinogen contents, however, remained unaltered in ulcer-prevented group, while in the ulcerated, were greatly reduced. It, therefore, appears that pyloric ligation alone does not cause that degree of adrenal insufficiency which may effect alteration in pepsinogen contents. Hirschowitz *et al.* (*loc. cit.*) further suggested that starvation may be another factor responsible for the reduction of gastric pepsinogen. In our experiments starvation equally affected all the groups, but the excessive reduction of pepsinogen contents was only marked in the ulcerated group which cannot be explained by starvation. Some other mechanism may, therefore, be involved in the reduction of pepsinogen.

Vineberg (1931) and Babkin (*loc. cit.*) observed that the stimulation of vagus or the mechanical stimulation of pylorus caused increased peptic activity. In our experiments it appears that pyloric ligation in ulcerated group of animals caused stimulation of vagus which brought significant decrease in the pepsinogen content, increase peptic activity in the gastric juice hence lowering of the pepsinogen peptic activity ratio. In six animals highly increased values of peptic activity were associated with deep ulceration and perforation showing a relationship between increased peptic activity and the severity of lesion. Vanzant *et al.* (1936) also observed similar correlation. In animals where ulceration was prevented peptic activity, however, remained within normal limits and pepsinogen-peptic activity balance maintained.

Hyperacidity has been considered by Mann (*loc. cit.*) and Grossman (*loc. cit.*) to have a direct corrosive action on the gastric mucosa resulting in ulceration. In our experiments in twelve animals in the ulcerated group hyperacidity above 30 m.eq./l. was associated with ulceration. In eight animals, hypoacidity less than 30 m.eq./l. was present and out of these five showed ulceration. In gastric ulceration following ligation of pylorus, hyperacidity appears to be related but not consistently to ulceration.

LeVeen (*loc. cit.*) sought to determine which constituent of the gastric juice, pepsin or the acid, was responsible for ulceration. He showed that hydrochloric acid did not damage the intestinal mucosa or made it more susceptible for the digestion of pepsin. The experiments of LeVeen indicated that increased peptic activity alone could cause intestinal ulceration, but did not establish its importance in relation to the development of gastric ulceration where the defence mechanism is superior than present in the ileum (Sanchez-Palomera, 1951). In the present experiments the factor or factors responsible to sensitize or bring about initial damage to the gastric mucosa may be vascular disturbance caused by the ligation of pylorus or the increased acidity of the gastric juice, but these studies clearly demonstrate a positive relationship between increased peptic activity and ulceration.

SUMMARY.

1. Acute peptic ulceration was produced in rats within sixteen hours after the ligation of pylorus. The site of ulceration was confined to the cardiac end of the stomach and the severity varied from mucosal hæmorrhage to deep ulceration.

2. Histochemical studies and estimations of the pepsinogen contents of the whole stomach were made and peptic activity and acid content of the gastric juice determined in normal (control), ulcerated and ulcer-prevented animals.

3. Pepsinogen in ulcerated group was significantly lower than in the normal animals. There was a significant increase of peptic activity in the ulcerated animals. The mechanism for the increase of peptic activity and decrease in the pepsinogen contents of the stomach has been discussed. A positive relationship between increase peptic activity and ulceration was present.

4. The role of hyperacidity in relation to peptic ulceration after the ligation of pylorus, has been studied and it has been noted that hyperacidity is related but not consistently to ulceration.

5. Protection from ulceration after pyloric ligation was brought about by the intraperitoneal injections of multiple doses of anticholinergic drug, oxyphenonium bromide. This procedure apparently helped to maintain normal pepsinogen and peptic activity balance.

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STUDIES ON THE COMPARATIVE UTILIZATION OF GLUCOSE, FRUCTOSE AND GALACTOSE IN ALLOXAN-DIABETES*.

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INTRODUCTION.

ALTHOUGH still controversial, the metabolic lesion in the utilization of glucose in diabetes is attributed primarily to an impairment in its initial phosphorylation to glucose-6-phosphate by hexokinase. Since the metabolism of other hexoses, such as fructose and galactose, seems to be initiated by different kinases (Slein *et al.*, 1950; Leloir, 1953), it would appear that these sugars might serve as metabolic fuels in diabetes. Fructose metabolism has often been studied in this content (Root *et al.*, 1946; Miller *et al.*, 1952; Smith *et al.*, 1953; Runyan and Kantor, 1954; Moorhouse and Kark, 1955). The results suggested that diabetics utilized fructose normally and better than glucose. Thus, in diabetic patients administered fructose was not only removed from the blood as readily as in normal persons but it was accompanied by lesser hyperglucemia and glucosuria and a prompt fall in the blood concentration of inorganic phosphate and relatively large increments in the levels of intermediary metabolites, such as pyruvate, lactate, etc., in contrast with the minor changes observed in these constituents after that of glucose. It has also been reported that fructose is oxidized at nearly normal rates by diabetic rat liver *in vitro* (Chernick and Chainkoff, 1951) and by the liver of diabetic patients *in vivo* (Caraig *et al.*, 1951). Comparative studies on the metabolism of galactose (Alslev, 1952) have shown that the blood galactose, pyruvate and lactate curves in diabetics are of the same magnitude and pattern as in normal subjects, although some elevation occurred in the blood glucose levels.

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The normal metabolism of these sugars in diabetes is explained on the basis that they enter the Embden-Mayerhof scheme at points different from that of glucose by the action of kinases, which unlike the one concerned in the case of glucose, mediate these reactions independently of insulin. However, once they join the glycolytic path, they could be converted into glucose-6-phosphate, and hence, in spite of the different metabolic routes followed initially, they could be potential sources of blood glucose. This seemed to be their main metabolic fate from a previous study (Banerjee and Divakaran, 1957) of their utilization in alloxan-diabetic rabbits. It was noticed that feeding of fructose and galactose to diabetic rabbits produced marked elevations in blood glucose levels, and consequently increased glucosuria, although there was no difference in blood fructose and galactose curves or in the urinary excretion of these sugars from that in normal rabbits. Consequently, when the over-all utilization of these sugars was compared with that of glucose, they appeared only slightly superior to it as metabolic fuels in diabetes.

Since intestinal mucosa and liver contain enzyme systems which could convert fructose into glucose (Stewart and Thompson, 1941; Bollman and Mann, 1931), it was thought that intravenous administration would be a more relevant procedure to assess the utility of these sugars as substitutes for glucose in diabetes. Accordingly, we have studied the comparative utilization of intravenously administered glucose, fructose and galactose in normal and alloxan-diabetic rabbits. The results show, as in the previous study, that in the diabetic organism fructose and galactose cause elevation of blood glucose, although there is no modification in the rate of their withdrawal from the blood stream.

EXPERIMENTAL.

Rabbits weighing 1 kg. to 2 kg. were used in the experiments. After serving as normal controls, they were made diabetic by intravenous injection of alloxan monohydrate (Banerjee, 1945). The animals were fasted 18 to 20 hours before administration of the sugars. A 50-per cent solution of each sugar, at the rate of 1 g. per kg. body-weight, was injected into the ear vein at a rapid rate. Blood samples were collected before and at $\frac{1}{2}$ -hourly intervals up to $2\frac{1}{2}$ hours after the administration of glucose and fructose and up to $3\frac{1}{2}$ hours after that of galactose. These were analysed for total sugar (Hagedorn and Jensen, 1923), inorganic phosphate (Fiske and Subba Row, 1925) and fructose (Roe, 1934) or galactose. Galactose was estimated by the Hagedorn and Jensen procedure after removal of glucose with freshly grown *Saccharomyces cerevisiae*. Glucose was computed as the difference between total sugar and the glucose equivalent of fructose or galactose depending on the sugar administered. The effect of fructose on blood glucose was also studied in both normal and diabetic animals given an intravenous injection of 0.4 mg. dihydro-ergotamine methane sulphonate prior to its administration.

Samples of 24-hour urine collected after the administration of different sugars were analysed for individual sugars in the case of normal animals and for total sugar as well in the case of diabetic ones. The methods used were the same as those employed for the estimation of these sugars in blood, except that the urine collected

prior to the administration of each sugar was used as a blank in its determination. Total sugar in the urine of diabetic rabbits was measured by Benedict's (1911) titration method. It was noticed that in fasted diabetic rabbits the increase in urine glucose over the previous day's value after the administration of each sugar exceeded even the entire amount administered. This indicated that the food consumed after the test contributed some glucose in the urine. Hence, the total as well as individual sugars excreted after the administration of different sugars by non-fasted diabetic rabbits receiving the same amount of food daily were also estimated, since this would be more appropriate to evaluate their comparative utilization.

RESULTS.

Subtraction of the glucose equivalent of fructose after its administration from the total sugar of the blood samples revealed that their reducing powers were far in excess of that of the fasting sample. Determination of the reducing power of these blood samples after treatment with freshly grown yeast showed that non-sugar reducing substances were only about 10 mg. per 100 c.c. of blood and that their concentrations did not vary at different intervals. Hence, it was assumed that the increases in reducing power of the blood, observed after fructose and galactose and which could not be accounted for by either of these sugars must be due to elevations in blood glucose. The mean increases in blood glucose levels thus observed after the administration of different sugars are shown in Table I. The magnitude of this rise varied with the sugar administered, but it was almost

TABLE I.

Blood glucose levels after administration of glucose, fructose and galactose in normal and alloxan-diabetic rabbits.

Rabbits.	Sugar.	Mean fasting blood glucose, mg./100 c.c.	MEAN INCREASE IN BLOOD GLUCOSE ABOVE FASTING LEVEL (MG./100 C.C.):				
			Hours after administration of sugars:				
			$\frac{1}{2}$	1	$1\frac{1}{2}$	$2\frac{1}{2}$	$3\frac{1}{2}$
Normal	(6) Glucose ...	92±6	193±20	132±24	77±25	8±11	
"	(6) Fructose ...	89±7	73±14	65±13	38±11	12±7	
"	(6) Fructose†...	101±5	29±10	28±9	9±6	-16±4	...
"	(6) Galactose ...	91±7	17±10	12±3	7±5	-2±2	...
Diabetic	(6) Glucose ...	303±47	208±14	173±19	145±24	109±33	-1±1
"	(6) Fructose ...	326±75	74±23	108±14	111±18	92±21	...
"	(6) Fructose†...	281±67	78±14	105±12	101±10	87±21	...
"	(6) Galactose ...	286±68	38±28	61±30	76±22	67±19	48±18

The figures in parenthesis indicate the number of rabbits.

* Standard deviation.

† Administration of fructose in these animals was preceded by injection of 0.4 mg. dihydro-ergotamine methane sulphonate.

identical initially for a given sugar in both normal and diabetic rabbits. Dihydro-ergotamine partly suppressed this elevation after fructose in normal animals but not in diabetic ones. The response of blood inorganic phosphate towards the three sugars was neither consistent nor significant. In normal rabbits fructose and glucose produced minor falls in blood phosphate, but in diabetic rabbits similar decreases occurred only after fructose (Table II). The levels of fructose and galactose in the blood of diabetic rabbits after the administration of the respective sugar did not differ from those of normal animals (Tables III and IV). The urinary excretion of these sugars also did not vary significantly between the two groups (Table V). The magnitude of the increase in urine glucose after each sugar corresponded empirically to the magnitude of the elevation in blood glucose it produced and to the severity of diabetes. Thus, the increase in urine glucose could account for 62 to 95 per cent of the amount administered in the case of glucose, 26 to 76 per cent in the case of fructose and 19 to 31 per cent in the case of galactose, besides 13 to 18 per cent of fructose and 18 to 30 per cent of galactose excreted as such (Table VI).

TABLE II.

Blood inorganic phosphorus levels after administration of glucose, fructose and galactose in normal and alloxan-diabetic rabbits.

Rabbits.	Sugar.	MEAN LEVEL OF BLOOD INORGANIC PHOSPHORUS (MG./100 C.G.):					
		Hours after administration of sugars:					
		0	$\frac{1}{2}$	1	$1\frac{1}{2}$	$2\frac{1}{2}$	$3\frac{1}{2}$
Normal	(6) Glucose	5.68 ± 0.64	5.32 ± 0.80	5.10 ± 0.78	5.23 ± 0.61	5.53 ± 0.72	...
"	(6) Fructose	5.57 ± 0.39	5.07 ± 0.55	5.25 ± 0.61	5.37 ± 0.54	5.47 ± 0.43	...
"	(6) Galactose	5.50 ± 0.54	5.48 ± 0.59	5.50 ± 0.54	5.52 ± 0.50	5.52 ± 0.50	5.43 ± 0.57
Diabetic	(6) Glucose	5.35 ± 1.11	5.35 ± 1.11	5.22 ± 0.99	5.17 ± 0.98	5.17 ± 0.98	...
"	(6) Fructose	4.87 ± 0.83	4.65 ± 1.00	4.77 ± 0.94	4.87 ± 0.83	4.88 ± 0.91	...
"	(6) Galactose	4.92 ± 0.72	4.92 ± 0.72	4.92 ± 0.72	4.92 ± 0.72	4.87 ± 0.75	4.68 ± 0.79

TABLE III.

Fructose levels in blood after administration of fructose in normal and alloxan-diabetic rabbits.

Rabbits.		MEAN LEVEL OF FRUCTOSE (MG./100 C.C. BLOOD):			
		Hours after administration.			
		$\frac{1}{2}$	1	$1\frac{1}{2}$	$2\frac{1}{2}$
Normal	(6)	88 ± 11	22 ± 5	6 ± 2	0
Diabetic	(6)	97 ± 9	21 ± 4	5 ± 3	0
Normal	(6)*	108 ± 11	24 ± 5	6 ± 2	0
Diabetic	(6)*	98 ± 10	22 ± 5	8 ± 2	0

* Administration of fructose in these animals was preceded by injection of 0.4 mg. dihydro-ergotamine methane sulphonate.

TABLE IV.

Galactose levels in blood after administration of galactose in normal and alloxan-diabetic rabbits.

Rabbits.	MEAN LEVEL OF GALACTOSE (MG./100 C.C. BLOOD):				
	Hours after administration.				
	$\frac{1}{2}$	1	$1\frac{1}{2}$	$2\frac{1}{2}$	$3\frac{1}{2}$
Normal (6) ...	134 ± 7	82 ± 11	44 ± 7	10 ± 7	0
Diabetic (6) ...	145 ± 12	89 ± 9	46 ± 7	6 ± 4	0

TABLE V.

Twenty-four hour urine sugars after administration of glucose, fructose and galactose in fasted normal and alloxan-diabetic rabbits.

Rabbits.	Sugar.	Average amount administered, g.	URINE SUGARS:	
			Average amount excreted, mg.	As percentage of amount administered.
Normal	(6) Glucose ...	1.270 ± 0.175	94 ± 24	7.4
"	(6) Fructose ...	1.240 ± 0.210	156 ± 42	12.6
"	(6) Fructose* ...	1.625 ± 0.260	172 ± 25	10.6
"	(6) Galactose ...	1.260 ± 0.200	306 ± 84	24.3
Diabetic	(6) Fructose ...	1.275 ± 0.244	152 ± 42	11.9
"	(6) Fructose ...	1.290 ± 0.210	119 ± 41	9.2
"	(6) Galactose ...	1.310 ± 0.170	381 ± 92	29.1

*Administration of fructose in these animals was preceded by injection of 0.4 mg. dihydro-crogtamine methane sulphonate.

TABLE VI.

Twenty-four hour urine sugars after administration of glucose, fructose and galactose in four non-fasted alloxan-diabetic rabbits.

Sugar.	Amount administered, g.	URINE SUGARS:			
		Glucose excreted before administration, g.	Glucose excreted after administration, g.	Amount excreted unchanged, mg.	Percentage amount utilized.
Glucose ...	1.250	4.042	5.029	...	20.9
Fructose ...	1.250	4.196	4.849	190	32.7
Galactose ...	1.250	3.877	4.190	326	49.0

DISCUSSION.

It is apparent from a comparison of the blood levels of different sugars after their administration in normal and diabetic rabbits that the latter withdraw fructose and galactose from the blood as rapidly as the former. This suggests the absence of any impairment in the initial stages of their metabolism in diabetes. This is in conformity with the findings of others (Root *et al.*, *loc. cit.*; Miller *et al.*, *loc. cit.*; Smith *et al.*, *loc. cit.*; Runyan and Kantor, *loc. cit.*; Alslev, *loc. cit.*) and is consistent with the concept that the metabolism of these sugars is initiated by enzymes distinct from the one involved in that of glucose and that these enzymes are uninfluenced by insulin. Yet, in spite of the prompt removal of these sugars, the reducing power of the blood after their administration is considerably enhanced. This rise in reducing power cannot be accounted for by either fructose or galactose and can be abolished by fermentation. Further, substantial increments also occur in the urine glucose in diabetic animals. Evidently, all these indicate an increase in the true glucose concentration of the blood. The most obvious explanation for this would be the conversion of fructose and galactose into glucose in the animal body, although it is conceivable that while fructose and galactose are being metabolized the organism is unable to make use of the glucose which it might be utilizing otherwise. But the increase in blood glucose after fructose and galactose in diabetic animals is much more than in normal animals and hence the latter interpretation seems to be incompatible.

The possible pathway whereby fructose and galactose are transformed into glucose might be as follows. Although the occurrence of fructokinase has been demonstrated both in liver and muscle (Slein *et al.*, *loc. cit.*), there are evidences to show that in the intact organism liver is the main site of fructose metabolism (Craig *et al.*, *loc. cit.*; Mendeloff and Weichsebaum, 1953; Wick *et al.*, 1953; Van Itali *et al.*, 1954). Galactokinase does not seem to be present in any other tissue except liver and possibly intestinal epithelium (Leloir, *loc. cit.*; Hele, 1950). Hence, in all probability liver might be the main organ concerned with the metabolism of fructose and galactose (Wick and Drury, 1954). Now, in the liver fructose enters the glycolytic pathway at the stage of fructose-6-phosphate (Cori *et al.*, 1951) and galactose at that of glucose-1-phosphate (Leloir, *loc. cit.*) and both these compounds could be converted into glucose-6-phosphate. Owing to the presence of glucose-6-phosphatase (Swanson, 1950), it is obvious how fructose and galactose could easily form blood glucose, even without passing through the stage of liver glycogen. Since the specific enzyme, glucose-6-phosphatase, seems to be absent in muscle (Cori *et al.*, 1938), it is unlikely that once fructose enters the metabolic stream in this tissue it can directly contribute any glucose into the blood. There are evidences to show that kidney might also be involved in the conversion of fructose into glucose (Bollman and Mann, *loc. cit.*); Griffiths and Waters, 1936; Reinecke, 1956). However, considering the important role of liver in the regulation of blood glucose, the elevated levels of glucose observed after the administration of fructose and galactose might be attributed to gluconeogenesis occurring mainly in this organ.

It appears from the initial magnitude of the rise in blood glucose after each sugar that this transformation occurs approximately to the same extent in both

normal and diabetic rabbits. The greater elevations seen at later intervals in diabetic animals might be due to their inability to utilize the glucose so formed. It has been reported (Langdon and Weakly, 1955) that glucose-6-phosphatase activity of the liver is increased in alloxan-diabetes. The enhanced activity of this enzyme might also be responsible for the abnormal elevations in blood glucose observed in diabetic animals. Other investigators (Root *et al.*, *loc. cit.*; Miller *et al.*, *loc. cit.*; Smith *et al.*, *loc. cit.*; Runyan and Kantor, *loc. cit.*) have reported only comparatively lesser increases in blood glucose after administration of fructose to diabetic subjects. However, a recent *in vitro* study (Renold *et al.*, 1954) with diabetic rat liver shows that in spite of the normal phosphorylation of fructose, a major portion of it is converted into glucose.

Although the amounts of fructose and galactose eliminated in the urine of diabetic animals are of the same magnitude as of normal animals, the administration of these sugars is followed by substantial increases in the urinary output of glucose in the former group. The concomitant glucosuria is commensurate with the increase in blood glucose caused by each sugar and the severity of diabetes. No doubt the magnitude of hyperglucemia and glucosuria observed in diabetic animals after the administration of fructose and galactose is comparatively less than that obtained after that of glucose, nevertheless, the advantage does not seem to be sufficient enough to advocate the use of these sugars as alternate metabolic fuels in diabetes.

SUMMARY.

1. The comparative utilization of three monosaccharides viz., glucose, fructose and galactose, administered intravenously was studied in normal and alloxan-diabetic rabbits.

2. Blood samples obtained before and at intervals after administration of these sugars were analysed the total sugar, inorganic phosphate fructose or a galactose. True blood glucose values were calculated by subtracting the glucose equivalent of fructose or galactose from the total sugar. Samples of 24-hour urine collected after the administration of different sugars were analysed for individual sugars in the case of normal rabbits and for total sugar as well in the case of diabetic animals. The 24-hour urinary output of glucose as well as fructose or galactose was also estimated after administration of the different sugars in non-tasted diabetic rabbits. The effect of dihydroergotamine on blood glucose was also studied by injecting 0.4 mg. of the substance into normal and diabetic rabbits prior to administration of fructose.

3. Diabetic animals removed fructose and galactose from the blood as readily as normal animals. The amounts of these sugars eliminated in the urine were not significantly different in the two groups. Obviously the initial stages of the metabolism of these sugars proceed normally in diabetes and that insulin does not mediate the actions of fructokinase and galactokinase.

4. However, administration of fructose and galactose was accompanied by elevations of blood glucose, which were of considerable magnitude in diabetic animals. Commensurate with this increase in blood glucose and the severity of diabetes,

there was enhanced glucosuria. Injection of dihydroergatamine partly suppressed the elevation of blood glucose after fructose in normal rabbits, but not in diabetic ones. The rise in blood glucose occurring after administration of fructose and galactose might be due to conversion of these sugars into glucose probably by the liver. The abnormal increase seen in diabetic animals might be either due to their inability to utilize the glucose so formed or due to enhanced rate of conversion.

5. When the over-all utilization of fructose and galactose by diabetic animals was assessed, they appeared to be assimilated only slightly better than glucose. Hence, the superiority of these sugars over glucose as metabolic fuels in diabetes does not seem to be much.

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HIGHER NERVOUS CONTROL OVER FOOD INTAKE*.

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THE rôle of central nervous system in regulating food intake has been long recognized, since either obesity or emaciation may occur in patients with nervous diseases. Interest has been focussed on the hypothalamic region as a result of the experimental studies of many workers (Hetherington and Ranson, 1940, 1942; Hetherington, 1941; Brobeck, Tepperman and Long, 1943; Kennedy, 1950) who have reported the development of obesity in rats associated with hyperphagia after bilateral lesions in the ventro-medial nucleus of the hypothalamus. Recent studies in rats, cats, and monkeys by Anand and Brobeck, 1951; Delgado and Anand, 1953; Anand, Dua and Shoenberg, 1955; and Anand and Dua, 1955, have shown the presence of two opposing regions of the hypothalamus regulating food intake; a 'feeding centre' in the lateral hypothalamus and a 'satiety centre' in the medial hypothalamus. These probably act as facilitatory and inhibitory mechanisms respectively for feeding reflexes mediated from a lower level. In the case of monkeys (Anand, Dua and Shoenberg, *loc. cit.*) results also suggested the existence of 'higher' control over these hypothalamic centres.

Bruce and Kennedy (1951) on the basis of their experiments with rats have suggested two distinct types of urges to eat; (a) *hunger*, the central regulator of which is located in the hypothalamus; (b) *appetite*, the central regulator of which is situated in the cerebral cortex and the influence of which may modify, in some cases even reverse, the effect of the hypothalamic influence. Pribram and Bagshaw (1953) reported in monkeys that temporal polar-amygdaloid formations are related to regulation of food intake. The present study was, therefore, undertaken to

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determine the relation of some of the 'higher' nervous regions to food intake. Since some structures in the frontal and temporal lobes (limbic system) are already known to influence certain visceral activities and affective behaviour, lesions were made in these regions to study their effects on food intake.

METHODS.

Forty-one monkeys (*macacus*) and thirteen cats survived following lesions in the frontal or temporal lobes. These were fed *ad libitum*, both before and after operation. Daily records of their food intake and weight were kept. They were observed for about a month before operation. The monkeys were fed on grams, nuts and fresh vegetables, and the cats on minced meat and milk. Intraperitoneal sodium pentobarbitone (M. & B.) was used for anaesthesia in each case; 0.7 to 0.8 c.c. of a 5 per cent solution/kg. body-weight in monkeys and 0.8 to 1 c.c./kg. body-weight in cats.

Bilateral surgical lesions were made by aspiration through large craniotomy holes in twenty eight monkeys and eight cats. In sixteen monkeys these involved different areas of the frontal lobes, in eleven monkeys different regions of the temporal lobes, and in one monkey these were in the occipital lobes. Seven cats had surgical lesions in the frontal lobes and one cat in the temporal lobes. Bifrontal lobectomies were carried out in one stage operation, while bitemporal lobectomies were done in two stages. Earlier lesions involved extensive areas of either the frontal or temporal lobes. In the latter studies, these lesions were restricted to small localized regions so that the relative effects of lesions in different regions on food intake could be studied.

For a similar reason in the remaining animals (13 monkeys and 5 cats), restricted bilateral electrolytic lesions were made stereotaxically in the following regions, by the technique described by Anand, Dua, and Shoenberg (*loc. cit.*).

			Number of monkeys	Number of cats
(i)	Orbital surface of the frontal lobes	...	3	1
(ii)	Anterior cingulate gyri	...	2	1
(iii)	Amygdaloid nuclei	...	4	2
(iv)	Hippocampus	...	2	...
(v)	Periamygdaloid region including pyriform cortex		2	1

The animals were kept alive for about 2 months after operation and finally sacrificed. Some of them died earlier. Macroscopic and histologic studies were made on their brains to localize and to assess the extent of lesions.

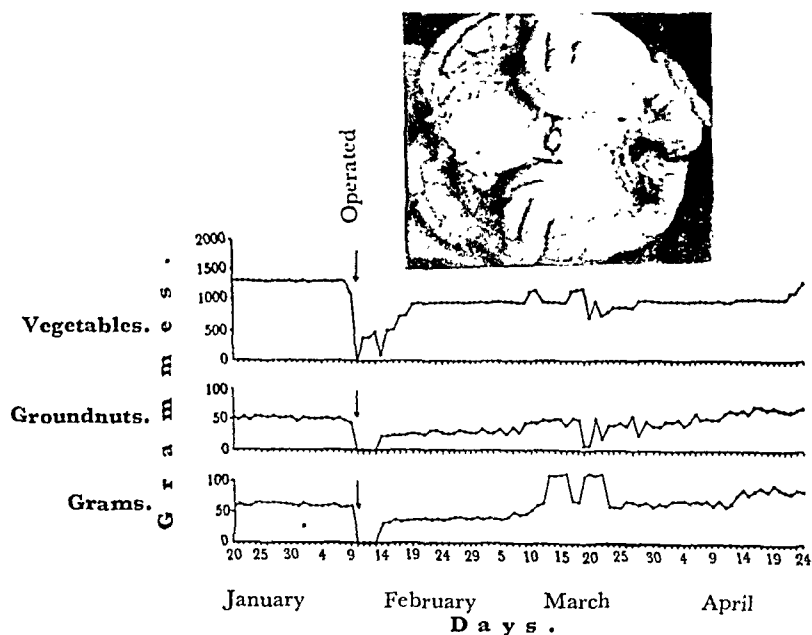
RESULTS.

The modifications in food intake which follow any of these lesions were only slight, and never so pronounced as those occurring after hypothalamic lesions (Anand and Brobeck, 1951; Anand, Dua and Shoenberg, *loc. cit.*).

Monkeys:

Frontal lobe lesions.—Lesions made either surgically or electrolytically of the frontal lobes were in twentyone monkeys. Sixteen had surgical lesions involving the frontal lobes. In six of them the frontal lesions were very extensive and involved in addition to the prefrontal lobes the posterior orbital surface and the anterior cingulate gyri (Graph 1). In these the total food intake decreased. Initially, the intake of all food stuffs was decreased, but later the intake of nuts and grams gradually returned to normal, whereas the vegetable intake remained low. Three of the six monkeys developed marked hyperactivity post-operatively, and the initial drop in food intake in these, although definite, was not so marked as in the other three. In these after some time the intake of grams and nuts even rose somewhat higher than the pre-operative level, but the vegetable intake remained low (Graph 1). Since the hyperactivity *per se* would have been expected to cause an increase in food intake, the decrease in intake assumes greater significance. The animals showing hyperactivity also had involvement of the head of the caudate nucleus.

GRAPH 1.

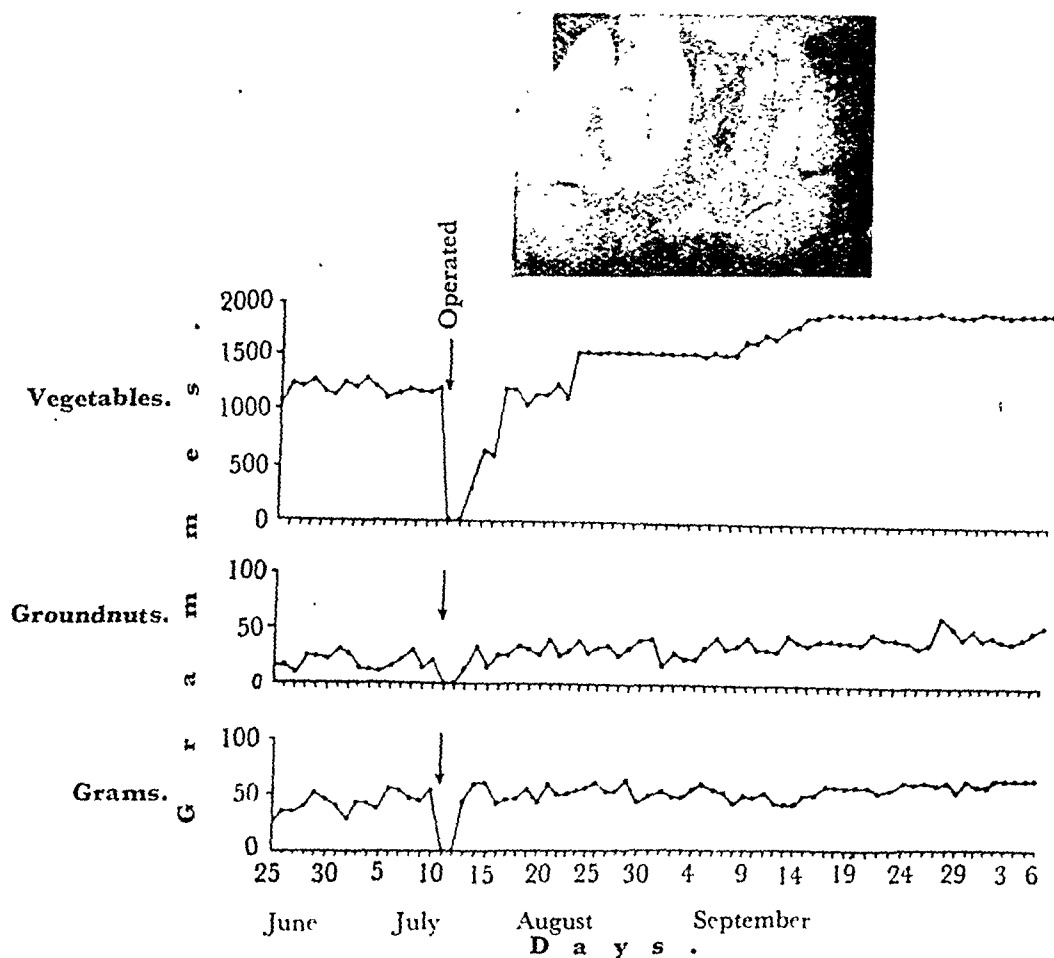


Food intake chart and brain photograph of monkeys 21, after extensive surgical lesions of frontal lobes including the posterior orbital surface. This monkey developed hyperactivity post-operatively.

In four monkeys the surgical lesions of the frontal lobes were less extensive and involved mainly the anterior tips, leaving the orbital cortex and anterior

cingulates intact. These showed a general increase in the intake of all food stuffs (Graph 2). Only one of these monkeys developed hyperactivity and, therefore, the increase in food intake could not be due to this.

GRAPH 2.

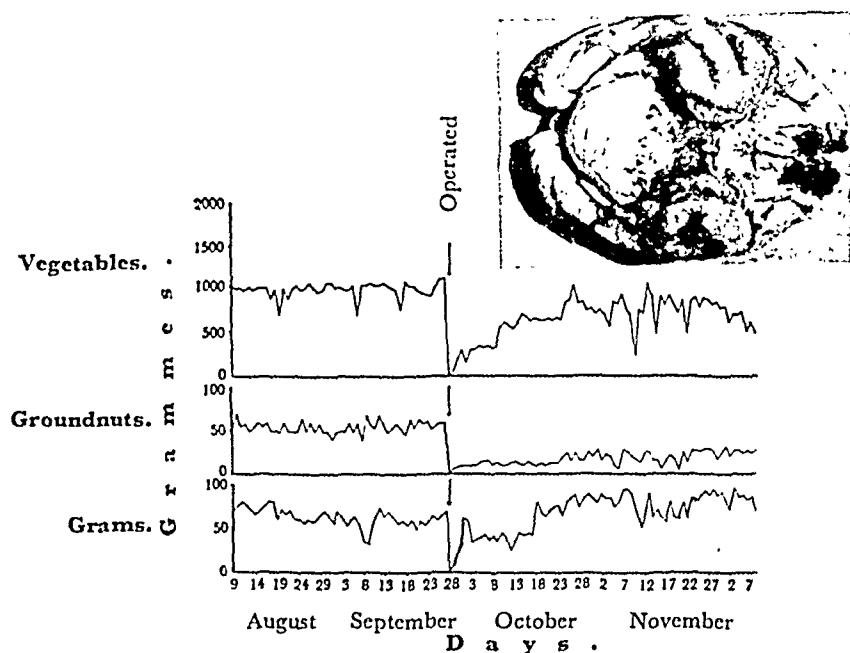


Food intake chart and brain photograph of monkey 2, after less extensive surgical lesions of frontal lobes (mainly the frontal tips) excluding the posterior orbital surface. There was no hyperactivity.

One monkey had surgical lesions restricted only to the posterior orbital cortex, and three more had electrolytic lesions involving this region. All of them developed a decrease in food intake (Graph 3), more marked for vegetables and groundnuts. The intake of grams tended to return to pre-operative levels soon after operation, while the intake of vegetables and nuts remained low. None of them showed any hyperactivity (*see* Fulton, 1951).

Two monkeys had surgical lesions and two others electrolytic lesions restricted to the anterior cingulate gyri. One of them with surgical lesions had some destruction of the adjoining medial frontal cortex too, and this showed a slight decrease

GRAPH 3.



Food intake chart and brain photograph of monkey 29, after surgical lesions restricted to the posterior orbital cortex.

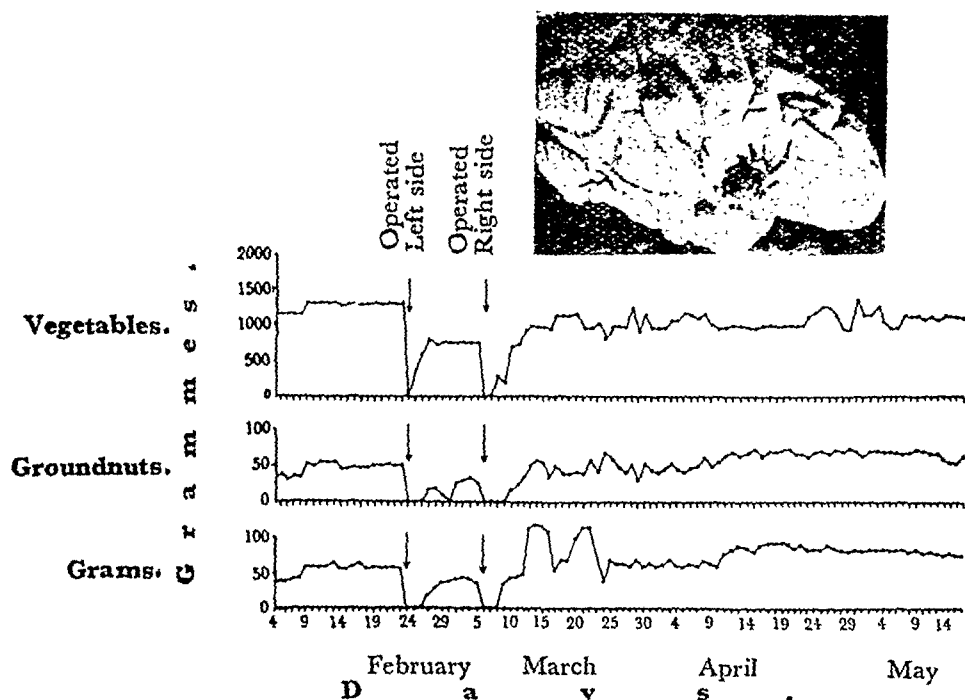
in food intake, mainly nuts, for a few days only. The remaining three failed to show any change in food intake.

Three other monkeys became comatose after extensive surgical frontal lesions. These were completely aphagic and died within a week. On post mortem they showed marked hæmorrhage into the frontal lobes.

Temporal lobe lesions.—Nineteen monkeys had lesions in the temporal lobes, made surgically in eleven and electrolytically in eight. Two monkeys had surgical lesions extensively involving the temporal lobes, including the amygdaloid and periamygdaloid regions. These showed a fall in the food intake for some days, before it gradually returned to the pre-operative levels and ultimately increased above this level (Graph 4). In two others, the surgical lesions, although extensively involving the temporal lobes and uncus, were more superficial and there was no involvement of the amygdaloid nuclei or the periamygdaloid regions. They had a general increase in food intake from the beginning (Graph 5).

In five other animals, the surgical lesions were slight and restricted to small temporal cortical regions. Two had mainly involvement of the temporal tips and these showed some increase in food intake. One had lesions of the insula and the adjoining temporal cortex and this also had some increase of food intake. Two others had involvement of the cortex on the lateral surface in the periamygdaloid region and these showed some decrease in food intake.

GRAPH 4.



Food intake chart and brain photograph of monkey 22, after extensive surgical lesions of the temporal lobes, including the amygdaloid regions.

All the five animals with lesions (electrolytic in four and surgical in one) restricted to the amygdaloid nuclei stopped eating for about 1 to 2 weeks during which time they were kept alive by stomach feeds (Graph 6). After this their food intake generally returned to normal levels. Three animals with lesions (two electrolytic and one surgical) of the anterior hippocampus and two animals with electrolytic lesions of the periamygdaloid regions (pyriform area) behaved similarly, with the difference that the aphagic period lasted for first 3 to 4 days only.

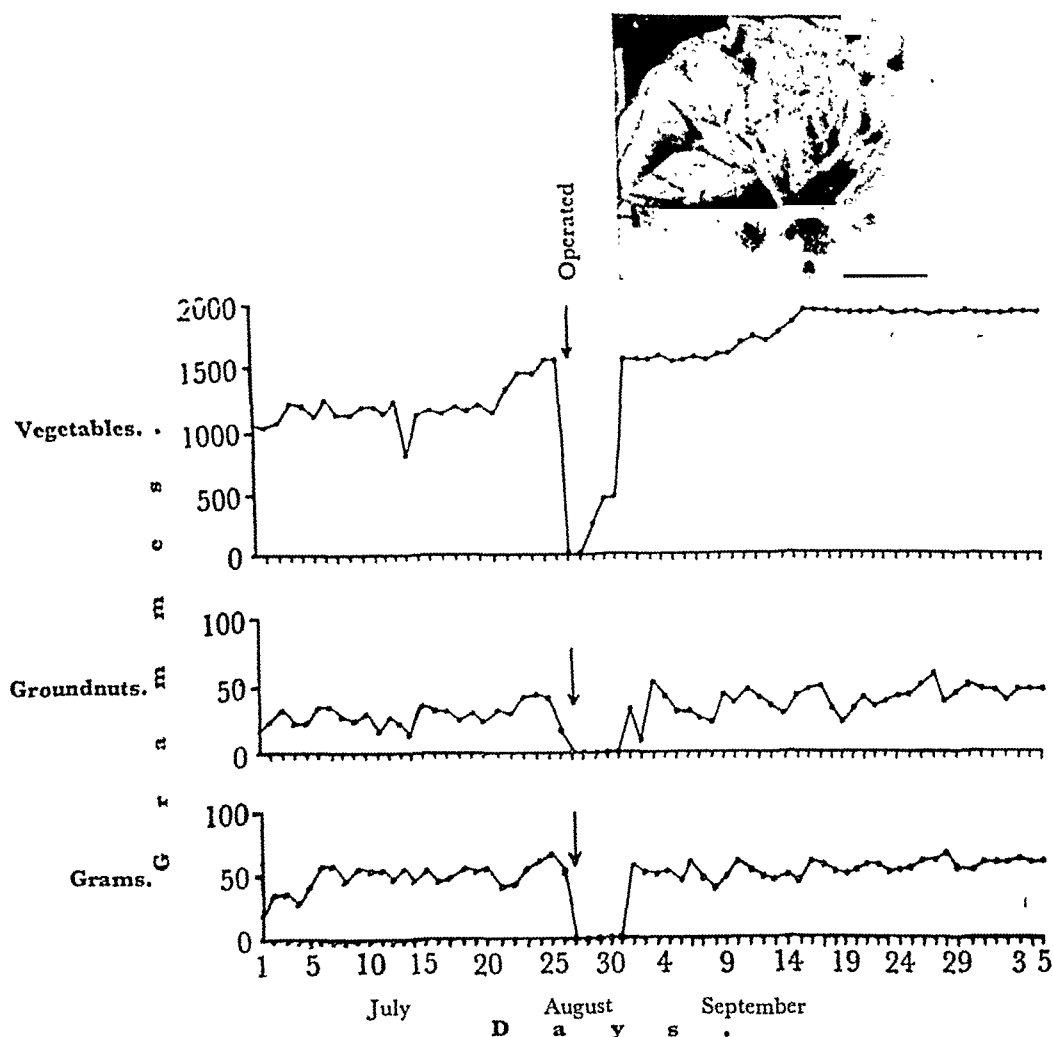
In none of the animals there was change in the water intake, in spite of the variations in the food intake.

Cats:

The quantitative changes in the food intake observed in cats after frontal and temporal lesions were much less than in monkeys after such lesions. An interesting observation in them was that the daily variations in the quantity of food intake which were marked in some cats, almost disappeared post-operatively and the daily intake became more or less constant.

Frontal lobe lesions.—Five cats had extensive surgical lesions involving the frontal lobes, but only two of them showed a slight decrease in the meat intake after the operation. One cat who had electrolytic lesions restricted to the orbital

GRAPH 5.

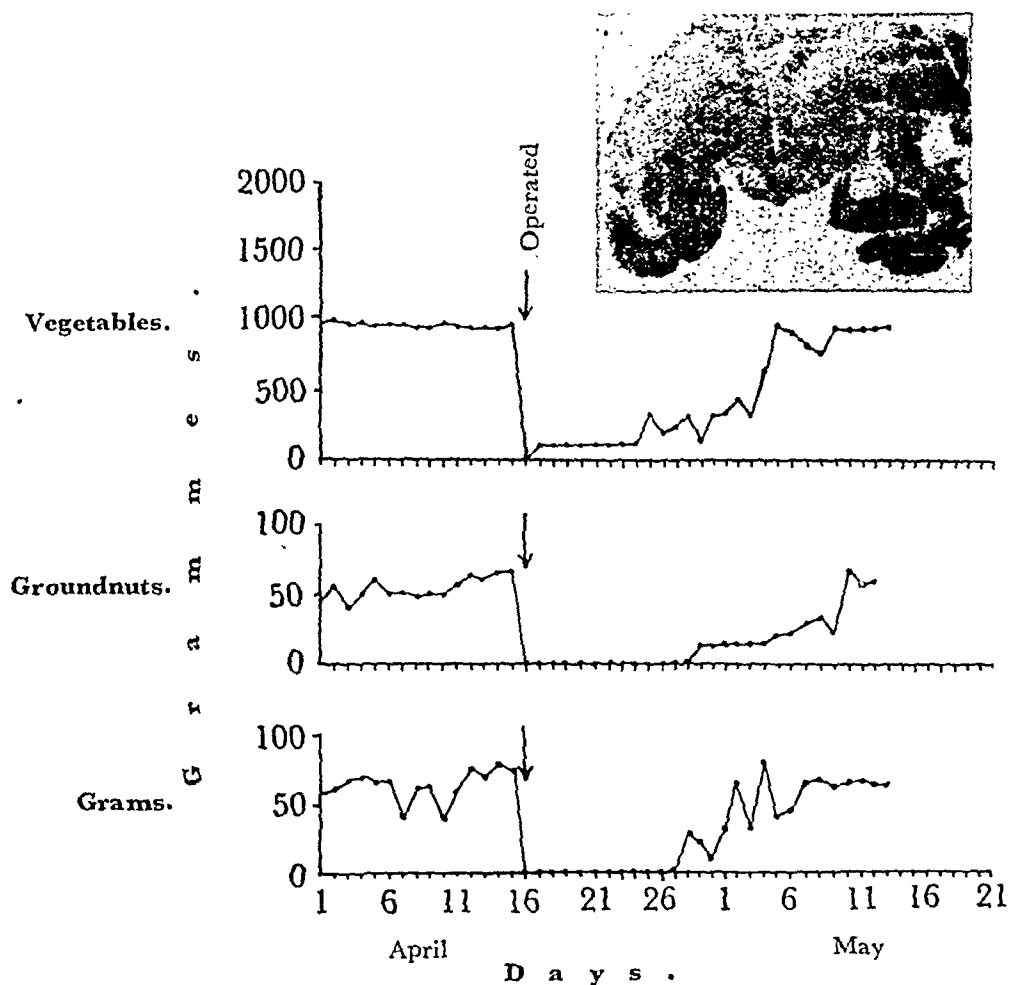


Food intake chart and brain photograph of monkey 3, after extensive superficial surgical lesions of the temporal lobes excluding the amygdala.

cortex, had low food intake for a few days only. Two cats had surgical lesions and one electrolytic lesion in the anterior cingulate. These did not show any change in food intake.

Temporal lobe lesions.—There was extensive surgical involvement of these lobes in one cat, and this showed a slight increase in the daily meat intake. Of the two cats with electrolytic lesions in the amygdaloid nuclei, one developed aphagia for about a week, ultimately returning to normal, and the other one had slightly decreased food intake for a few days which returned to normal. Another one had electrolytic lesions in the periamygdaloid region and showed decrease

GRAPH 6.



Food intake chart of monkey 28, after electrolytic lesions were given during the aphagic period. Section through amygdaloid nuclei shows electrolytic destruction of antero-medial group of amygdaloid nuclei.

in food intake for a few days. Aphagic and hypophagic responses in cats after amygdaloid lesions have also been reported in a previous study (Anand and Brobeck, 1952) although it was thought at that time that these may have been due to encroachment of the lateral hypothalamus.

It was also observed (Anand and Brobeck, 1952) that electrolytic lesions in the amygdaloid nuclei of the rats did not produce any change in their food intake.

DISCUSSION.

These experiments establish the control of forebrain structures over the mechanism of food intake. Previous studies (Anand and Brobeck, 1951; Delgado and Anand, *loc. cit.*; Anand, Dua and Shoenberg, *loc. cit.*; Anand and Dua, 1955) showed the presence in the hypothalamus of two opposing mechanisms for the

regulation of food intake, a 'feeding centre' in the lateral hypothalamus, and a 'satiety centre' in the medial hypothalamus. Bilateral destruction of the 'feeding centre' in the rat and cat produced a picture of complete aphagia leading to death by starvation. On the other hand, bilateral destruction of this region in monkey produced a slightly modified response (Anand, Dua and Shoenberg, *loc. cit.*). Such a monkey would not eat the food available in the cage but if it was put directly into its mouth it would swallow. Cat and rat would reject the food even when it was put into the mouth of the aphagic animal. It was presumed that this difference in the case of monkey was due to higher encephalization, and so the control over food intake, at least in primates, was also mediated through 'higher' centres.

Bruce and Kennedy (*loc. cit.*) have also concluded from their experiments on rats with hypothalamic lesions, that there are two types of urges to eat; one which is located in the hypothalamus named as 'hunger', and the other located in the cortex of the brain named as 'appetite'. The cortical influence may modify, or even reverse, the effect of the hypothalamic factor. Also the modification of food intake through cortically mediated impulses would not be expected to have such pronounced effects as the more primitive hunger (Kleiber, 1956).

Recent studies by a large number of workers have shown that frontal and temporal lobe structures belonging to the limbic system and the pre-frontal cortex, have a marked influence over certain visceral activities. These regions have, therefore, been designated collectively as 'visceral brain' (MacLean, 1949). It may, therefore, be argued that higher nervous control over food intake may also reside in these regions. These regions, therefore, have mainly been investigated in the present study.

The results indicate that although the modifications produced in food intake after frontal and temporal lesions are of a similar nature, they are much more marked in monkeys than in cats. This would be expected as the encephalization in monkeys is much higher than in cats. This would also explain why there was no change in food intake after amygdaloid lesions in rats (Anand and Brobeck, 1952). The disturbances in food intake were never so pronounced as after hypothalamic lesions, and also after these lesions in cats the daily variations in food intake, which were quite marked in some animals, usually disappeared. These facts would support the concept of 'primitive hunger' being located at the hypothalamic levels and 'discriminating appetite' in the brain. It is also interesting to note that brain lesions in monkeys affected the vegetable intake much more than the intake of grams and nuts which mainly supplied the calories.

Frontal lobe lesions which involved the posterior orbital cortex, either alone or along with other structures, usually led to a decrease in food intake which tended to return to, or exceed, the normal after about a month in spite of the hyperactivity occasionally produced. On the other hand, frontal lobe lesions which spared the posterior orbital surface developed an increase in food intake, even without hyperactivity. Lesions restricted only to the anterior cingulate did not change the food intake. Specific frontal lobe studies changing the food intake have not, to our knowledge, been reported, but in human beings both increase and decrease in food intake after lobotomy operations have been described.

The food intake tends to come back to its original levels, as the hypothalamic hunger or primitive urge to eat is intact, and only the discriminating mechanism is gone.

Lesions producing large defects in the temporal lobes, usually produced an increase in the food intake. If the amygdaloid region was spared, the increased intake started immediately after. If the amygdaloid region was also included in the damage, the food intake decreased for a few days, and later rose to more than the pre-operative level. Lesions restricted to the amygdaloid and periamygdaloid regions produced a marked decrease in food intake, even aphagia, for some days before the food intake returned to normal. Less extensive lesions further removed from amygdaloid region produced a small increase in food intake. Pribram and Bagshaw (*loc. cit.*) in monkeys, Rosvold, Fuller and Pribram (quoted by Fulton, 1951) in dogs, and Primbram, Epstein and Bagshaw (quoted by Fulton, 1951) in baboon, have all reported increased food intake after large surgical lesions involving this region. Anand and Brobeck (1952) have reported temporary aphagia in cats after lesions restricted to amygdala.

The controlling influence of the frontal and temporal regions over feeding behaviour is thus clearly established. The orbital surface of the frontal lobes and the amygdaloid complex are probably facilitatory regions, while inhibitory influences may also be passing down from the frontal and temporal regions. It may be mentioned here that stimulation of these regions in unanaesthetized animals has been shown to produce movements of 'eating' automatisms (Anand and Dua, 1956), i.e. chewing, licking, sniffing, sneezing, repetitive opening and closing of mouth with protrusion of tongue, but without any increase in food intake.

Davis (quoted by Fulton, 1951) has shown that hyperactivity after frontal lesions is due to involvement of the caudate nucleus rather than posterior orbital cortex. We found the same in this study. All the monkeys showing hyperactivity had damage to the head of the caudate nucleus, while some who had localized lesions of orbital cortex, did not show any hyperactivity.

SUMMARY.

1. Bilateral surgical lesions, some extensive and some restricted, involving different regions of the frontal or temporal lobes; or electrolytic lesions restricted to the orbital surface of the frontal lobes, anterior cingulates, amygdala and periamygdaloid regions, or hippocampus were made in forty one monkeys and ten cats and their effects on food intake studied.

2. Frontal lobe lesions including or restricted to the posterior orbital cortex, led to a decrease in food intake; while those which spared the posterior orbital cortex were followed by an increase in intake. Lesions restricted to anterior cingulate did not change the food intake.

3. Lesions in the amygdala and periamygdaloid regions caused aphagia or marked decrease in food intake lasting for a few days, after which the intake returned to normal levels. Extensive temporal lobe lesions were followed by an increase in food intake, preceded by an initial decrease if amygdala also was involved. Changes in food intake tended to disappear after some days.

4. Such changes were more marked in monkeys than in cats, and were never so pronounced as those associated with lesions of the hypothalamus.

5. Pre-operative daily variations in food intake tended to disappear following such lesions in cats.

6. It is concluded that frontal and temporal lobe structures modify food intake through a discriminating mechanism (*appetite*), which also influences the primitive urge (*hunger*) originating at the hypothalamic levels.

The authors acknowledge with thanks the help and guidance of Dr. Baldev Singh in this study.

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THE PROBLEM OF STANDARDIZATION OF LIVER PREPARATION*.

ISOLATION AND ESTIMATION OF SOME OF THE TOXIC CONSTITUENTS.

Part I.

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[Received for publication, July 29, 1957.]

WITH a view to lay down a specific method of standardization of liver preparations, investigations on the nature of the active principles of liver were being followed in this Laboratory (Dutta, 1955, and 1955*a*) during the last few years.

Attempts to prepare a whole liver preparation containing all the known essential active principles, but freed from all toxic as well as unwanted ingredients, have revealed the presence of some of the toxic substances in many preparations. Estimation and elimination of these unwanted substances seem to be of considerable significance in laying down a standard for such preparations.

Assuming that majority of the toxic substances in whole liver preparations might be of protein source, primary attempt was made to estimate protein and proteoses correctly in such preparations (*cf.* Dutta, 1956 and 1956*a*). Among the protein-degraded bodies, histamine might be in abundance and for micro-estimation of histamine a simple physico-chemical method applicable to any protein hydrolysate or biological fluid, has been devised (Dutta, 1956*b*). In our search for other toxic substances resulting from degradation or fragmentation of protein molecule, we have been able to show that besides protein and proteoses, a basic toxic polypeptide, and three amines—histamine, tyramine and tryptamine, are present in whole liver preparation. Detection and estimation of these toxic bodies by a physico-chemical method have been described in this paper.

* Abstract appeared in the *Proc. Ind. Sci. Cong.*, Part III, p. 433 (1957).

MATERIALS.

Four types of whole liver preparations were made:—

- (a) Raw ox liver was homogenized with a 'Premier high speed corrugated colloid mills' and extracted with water at 100°C.
- (b) Raw ox liver, homogenized as above, was extracted with 0.15 M sodium-chloride solution at room temperature (30°C.).
- (c) Homogenized raw ox liver, prepared as above, was extracted with 1 M sodium-chloride solution at room temperature (30°C.).
- (d) Raw ox liver was proteolysed with papain by a modified method of Davis *et al.* (1943).

All the above preparations were freed from excess protein and proteoses and they could be administered intramuscularly without any serious toxic manifestations, but none of them was suitable for intravenous injection. All these preparations had the same total solid content (20 per cent), but while each c.c. of the preparations (a), (b) and (c), was equivalent to about 4 g. of raw liver tissue, that of preparation (d) was 2.5 g. only.

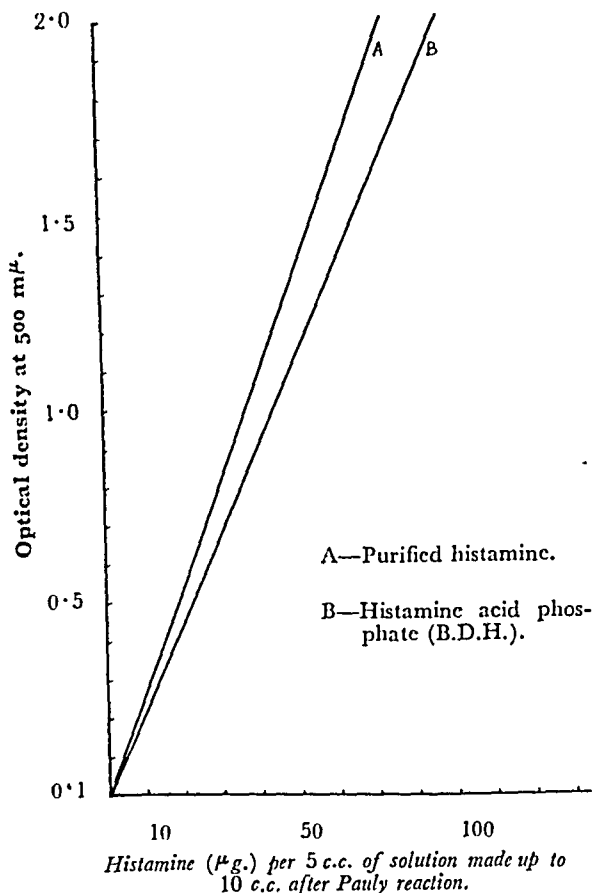
Preparation of standards of control.—The following amines were prepared in pure state:

(a) Pure histamine was prepared from 'histamine acid phosphate' (B.D.H.), either by refluxing with 4 per cent aqueous suspension of calcium carbonate or by percolating through a column of Dacelite F F (Permutit & Co., Ltd.) and the free base was further purified by chromatography using a solvent mixture of n-butanol, isopropanol and ammonia (10:15:5) in an atmosphere of ammoniacal butanol on a specially acid washed Whatman No. I paper. The pure histamine moved with Rf. 0.5 and other fractions had Rf. 0.0 and 0.73. The latter two fractions had no effect on blood pressure, but like histamine they formed a complex with diazotized sulfanilic acid. The pure histamine was eluted with water and estimated on the basis of nitrogen by micro-Kjeldahl method. The Graph shows the relationship between the Pauly-colour (Block and Bolling, 1951) and the amount of the base calculated on the basis of nitrogen content of the purified preparation of the histamine and the crude histamine acid phosphate (B.D.H.).

(b) Pure tyramine was prepared from tyrosine (Eastman Kodak Co., Ltd.) by heating with soda lime when the amine with other degraded products, such as phenol, etc., distilled over. The pure amine could be isolated from the distillate in two ways—either by adsorbing on Zeokarb 226 (Na form) and then eluting with 1.7 N aqueous ammonia or by chromatographing on a specially acid washed Whatman No. I paper with a solvent mixture of n-butanol, acetic acid, and water (45:5:10) when the amine moved with Rf. 0.53. The latter method was found to be preferable. The pure tyramine, thus obtained, was then estimated on the basis of nitrogen.

(c) Pure tryptamine was prepared in the same way as tyramine. The pure base here moved on the paper with Rf. 0.41. It was then estimated on the basis of its nitrogen content.

GRAPH.



(d) 5-Hydroxy tryptamine was prepared from a sample of 'enteramine' (Picrate of 5-hydroxy tryptamine). Free and pure base was obtained when 'enteramine' was chromatographed on Whatman No. I paper with a solvent mixture, used as in the case of tyramine, the base moving with Rf. 0.41, while the free picric acid moved with Rf. 0.68. The base was then estimated on the basis of nitrogen as above.

It should be noted here that both tryptamine and 5-hydroxy tryptamine had the same Rf. on paper chromatography using various solvents. Thus, with another solvent mixture of isopropanol, pyridine and water (50:25:25) both tryptamine and 5-hydroxy tryptamine had the same Rf. (0.7) and the picric acid 0.95.

EXPERIMENTAL PROCEDURE AND RESULT.

Whole liver preparations were slowly percolated through a column of Zeokarb 226 (Na-form) at pH 8.0 followed by excess of double distilled water. The resin

was then eluted in two phases—first with 1.7N aqueous ammonia and then after washing the resin with double-distilled water, again eluted with 1 N hydrochloric acid. The eluates were concentrated under vacuum at 45°C. to small volumes. The first phase of eluate contained all the toxic amines. The second phase of eluate was neutralized with sodium hydroxide and dialysed against water when a toxic macro-molecular polypeptide remained inside the membrane. This polypeptide was obtainable only from proteolysed whole liver preparations (Dutta, 1956a).

The toxic amines of whole liver preparation could also be isolated by another method. The charcoal (Dacro K-B) was found to adsorb these amines which could be eluted with a mixture of ethanol, water and hydrochloric acid (38:1:1). The eluate was concentrated under vacuum at 45°C. and the residual hydrochloric acid was neutralized with sodium hydroxide and centrifuged to discard the precipitated polypeptides. The clear supernatant solution contained all the above amines.

The above amines, as isolated by the above two methods, were free amines. There might be present some conjugated forms of them in the whole liver preparations and in order to obtain them in free conditions electrodialysis was found to be suitable. About 200 c.c. of the whole liver preparation was diluted with equal volume of distilled water and put in the central chamber of the dialyser, and the other two chambers containing the platinum-gauze electrodes were filled up with double-distilled water (400 c.c. each). There were two parchment papers in between these three chambers. The whole apparatus was put in ice-bath in order to maintain the temperature at 5°C. to 10°C. The current was maintained at 0.5 amp. for 16 hours. When the electrodialysis was completed the amperage came down to zero. The cathode chamber contained all the toxic amines, while the solution of the anode chamber was found to contain no such bodies. The residual solution in the central chamber contained only the macro-molecular bodies.

A suitable method of isolation of the total toxic amines was found to be as follows: a whole liver solution was electrodialysed and the liquid of the cathode chamber was percolated through Zeokarb 226 (Na form) at pH 8.0 and the resin then eluted with 1.7 N aqueous ammonia. The eluate was then concentrated under vacuum.

The resin eluate, containing either only the free amines or the total amines (free as well as those liberated from conjugated forms), was further fractionated on Whatman No. I paper. Histamine was characterized and estimated by the method of Dutta (1956b). Tyramine was isolated on paper using the same solvent mixture, as was used in the preparation of standard tyramine, and characterized by its Rf. value and light absorption curves of the coloured solution with diazotized sulfanilic acid and finally by a study of the physiological properties. It was then estimated like histamine. But complexity arose with tryptamine and 5-hydroxy tryptamine, because both had same Rf. value and similar chemical as well as physiological properties. Both these substances produced pale colour with diazotized sulfanilic acid, and similar fluorescent colour with either a mixture of potassium dichromate and formaldehyde (Shepherd and West, 1950) or a mixture

of ninhydrine and acetic acid (Jepsen and Stevens, 1953) under ultraviolet light. Also, tryptamine (or 5-hydroxy tryptamine) could not be estimated like histamine or tyramine, because the Pauly colour produced was rather too pale. So, the base (either tryptamine or 5-hydroxy tryptamine or a mixture) isolated on the paper, using solvent as in the case of tyramine, was eluted and analysed by micro-Kjeldahl method on the basis of nitrogen.

The macro-molecular polypeptide obtained from the second phase of elution (with 1 N hydrochloric acid) was reported before (Dutta, 1956a). The eluate was neutralized with sodium hydroxide and dialysed to remove the salts and other materials and then concentrated to a small volume. The composition and some of its chemical characteristics also have been already reported (Dutta, 1956a). It was estimated by the method developed in this Laboratory (Dutta, 1956).

The analytical results from the above experimental work are presented in the Table. It should be noted here that the different liver preparations, used in this investigation, were of the same source. The results were an average of five analyses.

TABLE.

The amount of free and conjugated histamine, tyramine, tryptamine (or its derivative) and the basic polypeptide.

Liver preparation.	HISTAMINE, $\mu\text{g./g.}$ RAW TISSUE.		TYRAMINE, $\mu\text{g./g.}$ RAW TISSUE.		TRYPTAMINE, $\mu\text{g./g.}$ RAW TISSUE.		Polypeptide, mg./g. raw tissue.
	Free.	Conjugated.	Free.	Conjugated.	Free.	Conjugated.	
a	5.8	1.5	12.4	12.9	2.2	2.8	...
b	6.7	1.8	12.9	14.5	2.9	1.9	...
c	6.6	2.2	13.1	14.8	2.6	2.2	...
d	8.3	4.6	18.5	12.7	2.7	3.1	2.4

Physiological properties of the toxic substances.—The effects of the isolated amines on the blood pressure were recorded using a cat (male, 2.5 kg.) which was chloralosed and atropinized. In Figure 1 it might be noted that the whole liver preparation—*a* (PL_2), preparation—*c* (PL_3) and preparation—*d* (prolin—a proteolysed liver) have similar type of effect, first lowering and then raising the pressure. The liver preparation—*b* (L 27A), preparation—*c* (L 27B) and preparation—*d* (L 27C) were fractionated into two groups by dialysis: (i) micro-molecular fraction, and (ii) macro-molecular fraction. Each of the six different fractions thus obtained from the above three preparations were then percolated through Zeokarb 226 (Na-form) separately. None of them was found toxic on being injected into white albino mice (14 g. to 16 g.). Fig. 2 showed that none of them affected the blood pressure also. Liver preparation—*d* was then electrodialysed and the fractions in the three chambers were marked as LP(—), LP(±), and LP(+) according to the nature of the charges of the electrodes. These three fractions were also injected into the cat (Fig. 2), when it was noted that fraction LP(—) from the cathode chamber contained all the blood pressure affecting substances.

FIG. 3.

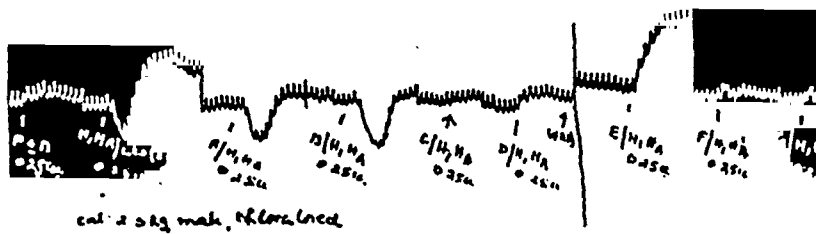


FIG. 4.

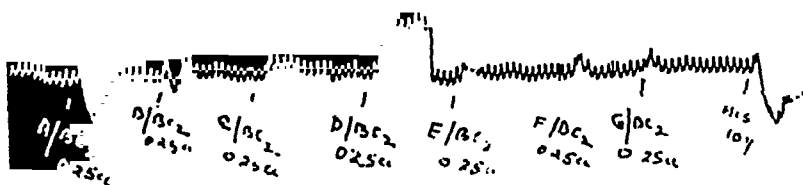
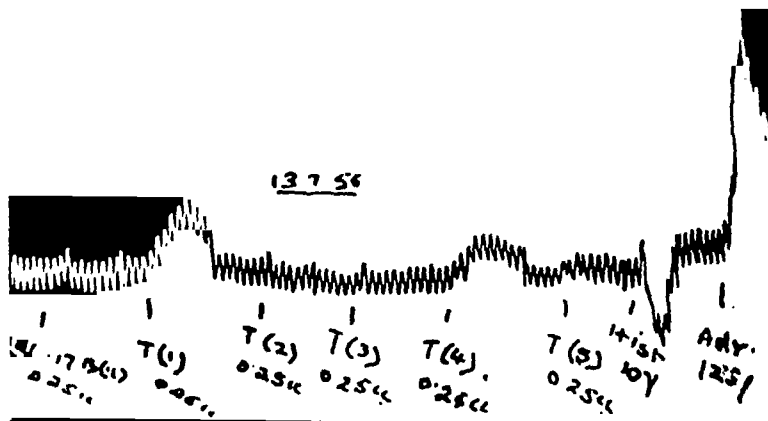


FIG. 5.



The fraction LP (—) from electrodialysis was also similarly analysed to find that the main blood pressure affecting bodies in the liver preparation were histamine, tyramine and tryptamine.

The polypeptide, isolated from the second phase of elution with acid from the Zeokarb 226 (Na-form), was found to produce toxic effects on being injected intramuscularly or subcutaneously into guinea-pig (400 g. approx.). There was first an inflammation, then induration and ultimately necrosis at the zone of injection. The toxic effect of the fraction of this nature was further confirmed by the following experiments. Whole liver preparation was precipitated with equal volume of ethanol in presence of 1 per cent calcium chloride, and the harvested precipitate was dissolved in 1 M sodium chloride solution and dialysed against running tap-water for 24 hours. The undialysable fraction was a hazy colourless solution containing all macro-molecular bodies in double concentration. This fraction on being injected intramuscularly produced toxic symptoms as above. Half of this solution was treated with Zeokarb 226 (Na-form) when it became non-toxic. However, the acid eluate of this resin, as purified before, produced similar toxic symptoms. The other half of the undialysable fraction was then treated with chloroform in presence of sodium chloride, and thus the macro-molecular polypeptides were removed after being denatured. The residual solution was again dialysed to remove the salts and when tested on guinea-pig, showed no toxic symptoms. So, it was apparent that some polypeptides, particularly of basic nature, should be removed from whole liver preparation.

Finally, we can report here a significant result of our experiments. All the liver preparations, (a, b, c, d) were tolerable when injected into animals intramuscularly, but intravenous injection (same dose) was lethal. When these preparations were treated with Zeokarb 226 (Na-form), they could be injected intravenously. So, apparently the resin adsorbed some of the unwanted fractions from the whole liver preparation.

DISCUSSION.

Four types of whole liver preparations were made and some unwanted toxic substances were isolated from each preparation. The liver preparations were electrodialysed when some of the toxic micro-molecular bodies accumulated in the cathode chamber, the anode chamber containing none of these toxic bodies. The electro-positive micro-molecular substances could be further purified by Zeokarb 226 and then finally fractionated by paper chromatography, when they were characterized as histamine, tyramine, and tryptamine and then estimated by physico-chemical methods. Among the macro-molecular bodies the main toxic body was characterized as a polypeptide of basic nature. There may be several other toxic bodies in whole liver preparations in micro-quantities which might not have been detected by the method used above. So, further investigations seem to be necessary.

Isolation and estimation of histamine from biological fluids have been recorded by many investigators. Thus, McIntire *et al.* (1947) estimated histamine in blood by a physico-chemical method, but this method was not found to work satisfactorily with liver preparations. Moreover, it was extremely difficult to

extract the base completely with n-butanol from the liver preparation. However, Ganguli and Bhattacharyee (1954) used n-butanol to extract histamine from the liver preparations. They used a resin to adsorb histamine from the preparations, but unfortunately this variety of resin was not found satisfactory in our hand as it was found to adsorb several other Pauly-positive materials also. Johnson's (1955) microchemical method also was not found to be suitable for a complex preparation like whole liver solution, although it might work satisfactorily in case of plasma. Robert and Adam (1950) and Horiuchi (1954) isolated histamine from biological fluid by adsorption on resin and then estimated it by biological methods. The resin could adsorb many other toxic bodies and so direct biological assay of the eluate might not give a correct picture on the amount of histamine present.

The significance of the method lies in the observation that it will not be affected by indole and phenolic acids often present in liver extracts (*cf.* Armstrong *et al.*, 1956).

Isolation and estimation of tyramine were easily carried out as in the case of histamine. But in case of tryptamine (or 5-hydroxy tryptamine) they were not so simple due to some technical difficulties, as reported in the text. The ultra-violet absorption of this fraction showed a band at 269-270 $m\mu$. It seems that the fraction might be a mixture of tryptamine and 5-hydroxy-tryptamine, and their accurate estimation would be of considerable significance. Estimation was done on the basis of nitrogen, so as to get an idea about the net amount of the nitrogenous base, in whatever form it might be present. Colorimetric estimation with p-dimethyl-amino-benzaldehyde or with 1-nitroso-2-naphthol could not also differentiate tryptamine and 5-hydroxy tryptamine in the mixture. This point deserves further investigation. It should be noted that the above method of estimating histamine, tyramine and tryptamine is not being affected by the presence of choline which is usually present in whole liver preparation. Choline has been estimated by another physico-chemical method which will be subject matter for a future communication.

It may be mentioned that proteolysed protein preparations are now often used in therapy. Naturally, isolation of toxic polypeptides as described in the body of the paper and a subsequent study on its nature and other characteristics would be of considerable interest.

Biological method for standardizing the toxicity of liver preparations is often standardizing being advocated (*cf.* Choudhury *et al.*, 1956). For preliminary screening work this might be helpful, but in case of actual estimation of each and every toxic substance that might be present, some physico-chemical method, as described in the body of this paper, may be of advantage. Further work is in progress to lay down a limit for these toxic bodies in liver preparations, specially meant for parenteral administration.

SUMMARY.

1. Physico-chemical methods have been described for isolation of free histamine, tyramine, tryptamine (or 5-hydroxy-tryptamine) and a basic polypeptide as well as for their estimation in different types of liver preparations.

2. These may be utilized for the estimation of any toxic amines present in conjugated form, after their dissociation by electrodialysis and subsequent isolation.

3. Certain physiological properties of the toxic bodies have been discussed and it has been shown that their isolation from the liver preparation is essential for full evaluation.

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COLORIMETRIC ANALYSIS OF PROTEIN BODIES.

BY

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ADVANTAGES of the use of chloroform and cetavlon (cetyltrimethyl ammonium bromide) for detection and estimation of protein and higher peptides have been recently reported from this Laboratory (Dutta, 1956 and 1956*a*). Since then, a further attempt was made to develop a simpler and finer technique using the characteristic ability of bromophenol blue (Schneider and Wunderly, 1952) (Tetrabromophenol sulfonphthalein) to dye the protein bodies, even in presence of polysaccharides, polynucleotides, lipides and various inorganic salts. By this method, native protein or higher polypeptides could be detected and estimated in presence of smaller protein degraded bodies. And also, the smaller protein degraded bodies could be similarly analysed in presence of native protein and higher polypeptides.

MATERIALS.

The following materials were required for the experimental work as described below:—

- (1) Protein sources:
 - (a) Normal horse serum.
 - (b) Egg white solution.
 - (c) Proteins of guinea-pig liver extracted with 0.9 per cent (in phosphate buffer, pH 7.0) sodium chloride.
 - (d) Proteins of guinea-pig liver extracted with 10 per cent (pH 6.24) sodium chloride.
 - (e) Proteins of guinea-pig liver extracted with 2 per cent sodium hydroxide solution.
- (2) Proteolysed goat meat solution (papain digested).
- (3) Proteolysed goat meat solution (papain and trypsin digested).
- (4) Bacto-peptone (Difco).
- (5) Glutathione (Eastman Kodak Co.).

- (6) Bromophenol blue (BPB), 0.5 per cent in methanol.
- (7) Aqueous calcium chloride solution (10 per cent).
- (8) Mercuric chloride.
- (9) Chloroform-octanol mixture (9:1).
- (10) Citrate buffer (Sorensen's), pH 6.75, containing methanol (30 per cent).
- (11) Aqueous sodium citrate solution (1 per cent).
- (12) Aqueous sodium thiosulfate solution (60 per cent).
- (13) Saturated ammonium sulfate solution.
- (14) Borax-borate buffer (Palitzsch), pH 8.5.

METHOD.

I. *Detection and estimation of native protein and higher peptides.*—A few drops of the test solution was put on a clean piece of filter paper, dried and sprayed with calcium-chloride solution and dried again. The paper was then sprayed with a bromophenol blue solution (0.05 per cent solution prepared from the stock solution with distilled water) and dried again. It was then washed slowly in water trough (pH 4.0 to 4.2) when the excess of dye, free as well as the portion reacted with smaller peptides, was washed off, leaving a distinctly stained spot of protein or higher peptides.

This spot was eluted with the citrate buffer at 37°C. for 16 hours and analysed by optical absorption at 595 m μ ., estimating the amount from the ratio $N_2 \text{ mg./c.c.}$

$\frac{E_{595}}{\text{m}\mu}$

II. *Detection and estimation of lower peptides.*—The test solution was freed from native proteins and higher peptides by treating with chloroform-octanol mixture and then a few drops of the test solution were put on a clean filter paper and dried up. The spot was sprayed with bromophenol blue (0.05 per cent solution prepared from stock solution with distilled water), saturated with mercuric chloride, dried, sprayed with ethanol and again dried. The paper was then washed in water when excess of dye (free) was washed out, leaving a stained spot for protein degraded bodies.

For quantitative analysis the spot could be eluted with citrate buffer, as before, and its optical absorption at 595 m μ . was measured.

EXPERIMENTAL WORK AND RESULT.

A. *Detection of proteins and higher peptides.*—An aqueous solution of native proteins was prepared from each of normal horse serum, egg white, and liver proteins, having the protein concentration about 100 $\mu\text{g. per c.c.}$ The solution was adjuvated with proteoses and peptones (from Bactopeptone), smaller peptides (from goat meat), and polysaccharides and polynucleotides (from goat liver), concentration of each adjuvant being about 1 per cent (w/v). The polysaccharide and polynucleotide were isolated according to modified method of Dutta *et al.* (1953).

It was analysed by Method (I), described above, using bromophenol blue with calcium chloride, when the native proteins produced distinct spots on paper. The dye combined with other protein degraded bodies are washed off. The polysaccharide and the polynucleotide did not react with dye at all.

B. *Detection of lower peptides.*—Similar solutions, as above, were prepared, the only changes being the increase of the concentration of the native protein to about 1 per cent and the decrease of concentration of the smaller peptides to about 0.01 per cent. These solutions were tested by Method (II), as described above, using bromophenol blue with mercuric chloride. Distinct coloured spots due to the smaller peptides were formed on the paper.

Even pure glutathione could be detected by Method (II).

A peptide, found to be consisting of five amino acids, was isolated from proteolysed liver solution. It could not be detected either by the chloroform-cetavlon method or by the bromophenol blue-calcium chloride reaction. It was, however, easily detected by the method using bromophenol blue with mercuric chloride.

C. *Quantitative estimation of protein and higher peptides as well as the lower peptides.*—Different proteins had different capacities to bind the dye (BPB), and the quantitative estimation of the dye by $E_{595} \text{ m}\mu$. would give the amount of the protein or polypeptide combined with it. The free dye as well as the dye combined with the protein bodies had always the maximum optical absorption (E) at 595 $\text{m}\mu$.

Egg white was mixed with excess dye (BPB) and then dialysed to remove the free dyes. The dyed protein was then analysed for its protein-nitrogen content according to a method reported before (Dutta, 1956), and for its $E_{595} \text{ m}\mu$. to evaluate the ratio $\text{N}_2 \text{ mg./c.c.}/E_{595} \text{ m}\mu$. as 2.07. But the ratio varied with various fractions of the same protein, as evident from the following experiment. The egg white solution (0.5 c.c.) was fractionated with ammonium sulphate solution at 25, 40, 50 and 75 per cent saturation in presence of sufficient dye (BPB) and subsequently centrifuged. Half of the supernatant solution (2 c.c.) of each fraction was diluted with citrate buffer (3 c.c.) and $E_{595} \text{ m}\mu$. recorded (Table I, A). The precipitated proteins were washed with 25, 40, 50 and 75 per cent saturated ammonium, sulphate solution, respectively, to remove the free dyes and then each precipitate was dissolved in citrate buffer (5 c.c.) and diluted with water to 10 c.c. and their $E_{595} \text{ m}\mu$. recorded (Table I, B). The protein-nitrogen of these solutions was also estimated to evaluate the ratio $\text{N}_2 \text{ mg./c.c.}/E_{595} \text{ m}\mu$. (Table I, C).

TABLE I.

Relation between the dye BPB with different fractions of egg white.

Ammonium sulphate, saturation per cent.	25	40	50	75
A	0.310	0.109	0.091	0.061
B	0.611	0.810	0.821	0.854
C	1.210	1.950	1.836	2.103

A similar experiment was done with normal horse serum. The serum, as a whole, was found to have the value of $\frac{N_2 \text{ mg./c.c.}}{E_{595} \text{ m}\mu.}$ as 1.10. It was then fractionated with sodium thio-sulphate at 24, 26, 28, 30 and 32 per cent concentration. The results are shown in Table II:—

TABLE II.

Relation between the dye BPB with different fractions of serum.

Concentration of $\text{Na}_2\text{S}_2\text{O}_3$, per cent.	24	26	28	30	32
A	0.740	0.479	0.478	0.130	0.090
B	0.151	0.417	0.418	0.764	0.788
C	1.81	1.46	1.46	1.24	1.17

It was thus apparent that each protein had its characteristic binding power for the dye (BPB). The quantitative binding power of different protein bodies was further verified by the following experiment:—

Goat-meat was proteolysed with papain to contain about 50 per cent amino nitrogen and seitzed. This clean and sterilized protein hydrolysate (A) was analysed. An aliquot (0.5 c.c.) of the solution was placed on a specially acid (dilute hydrochloric) washed filter paper and developed according to Method (I). The stained zone was cut out and eluted with citrate buffer (5 c.c.) and analysed for its $E_{595} \text{ m}\mu.$, and the nitrogen content (Table III, a), thus obtaining the values for higher polypeptides only. Then another aliquot (10 c.c.) was dialysed and readjusted to the original volume (10 c.c.). From this dialysed solution again 0.5 c.c. solution was analysed by Method (II) to get the values for total peptides (from the higher polypeptides to the lower tripeptides) (Table III, b). Then 5 c.c. of the dialysed solution was freed from higher polypeptides (including the proteoses) by treatment with a mixture of chloroform and octanol in presence of sodium chloride (0.3 g.) and then 0.5 c.c. of this fractionated solution was similarly analysed by Method (II) to get the values for lower peptides only (Table III, c).

TABLE III.

Relation between the dye (BPB) and the different fractions of protein hydrolysate (papain digested).

Fraction.	$E_{595} \text{ m}\mu.$	$N_2 \text{ mg./c.c.}$	$\frac{N_2 \text{ mg./c.c.}}{E_{595} \text{ m}\mu.}$
a	0.134	0.298	2.224
b	1.101	2.061	1.872
c	1.110	1.518	1.358
b—(a+c)	...	0.245	...

The N_2 value of $[(b) - (a + c)]$ might be accounted for the proteoses content in the protein hydrolysate.

The same papain digested protein hydrolysate was then further digested with trypsin and analysed just as above. The results were noted in Table IV:—

TABLE IV.

Relation between the dye (BPB) and the different fractions of protein hydrolysate (papain and trypsin digested).

Fraction.	$E_{595} \text{ m}\mu$.	$N_2 \text{ mg./c.c.}$	$\frac{N_2 \text{ mg./c.c.}}{E_{595} \text{ m}\mu}$.
a	0.031	0.063	2.010
b	1.125	1.985	1.764
c	1.488	1.826	1.227
$b - (a + c)$	...	0.096	...

Here also, the value of $[(b) - (a + c)]$ may indicate the amount of proteose in the protein hydrolysate. Every protein fraction thus had a quantitative relation between the dye (BPB) bound and its molecular structure, and so once that relation was evaluated, subsequent analyses of the optical absorption ($E_{595} \text{ m}\mu$) of the dyed protein bodies would give direct estimation of the substance.

Estimation of protein in a nucleoprotein.—From guinea-pig liver nucleoprotein was isolated using cetyl trimethyl ammonium bromide and then treated with excess ethanol to remove the surface active agent. The clear aqueous solution of the nucleoprotein was then analysed for its protein-nitrogen content by the method reported before (Dutta, *loc. cit.*), and also analysed according to the Method (I) described above, thus obtaining the ratio $\frac{N_2 \text{ mg./c.c.}}{E_{595} \text{ m}\mu}$ as 1.782.

Then, this nucleoprotein solution was gradually deproteinized in stages by treatment with chloroform-octanol mixture and in each stage the ratio was found to have the value about 1.782, the variation being ± 10 per cent only. Thus, it tends to indicate that the protein fraction of the nucleo-protein might be fairly homogeneous in nature.

D. *Effect of pH on the optical absorption of the dyed protein.*—Equal amounts (0.1 c.c.) of the nucleoprotein solution (isolated from guinea-pig liver) were put on pieces of filter paper and developed by bromophenol blue with calcium chloride as above and they were eluted with 5 c.c. of buffers, having pH from 5 to 12. The optical absorptions were recorded (*vide* Table V):—

TABLE V.
Effect of pH on $E_{595} \text{ m}\mu$.

pH:	5	6	7	8	9	10	11	12
$E_{595} \text{ m}\mu$.	0.230	0.238	0.242	0.249	0.251	0.255	0.264	0.285

E. *Nature of linkage between the protein and the dye (BPB).*—Several dyed-proteins were prepared and aliquots of each sample were treated as follows:—

(i) Sodium chloride was added to the solution (1 M concentration) and then shaken with chloroform-octanol mixture several times until no more gel was formed. It was noted that dyed proteins were dissociated, the dye remaining in the aqueous layer, while the protein separating out as the denatured colourless gel.

(ii) When the samples were subjected to paper ionophoresis under 220 V and 4 m A using borax-borate buffer (pH 7.0) containing excess sodium chloride (1 per cent), the dye was always found to dissociate from the protein and to travel fast towards the positive electrode, leaving the colourless protein behind.

F. *Stability of the protein-bromophenol blue.*—Dye-bound protein (egg white) was dialysed against running tap-water and at frequent intervals the value of the ratio $\frac{N_2 \text{ mg./c.c.}}{E_{595} \text{ m}\mu}$ was determined. It was noted that the ratio remained almost unchanged for 48 hours, after which its value gradually went up, indicating gradual dissociation. Dialysis against molar saline was, however, noted to cause a sharp rise in the value of the ratio.

Eluates of the stained spots with buffers were found to be stable even for four days.

DISCUSSION.

A common reagent used for detection and estimation of protein bodies is ninhydrin (Ruhemann, 1910). This reagent is very sensitive, but has got some drawbacks. It gives similar colour reaction from the highest polypeptide to the simple amino acids and so cannot practically differentiate the higher peptides from the lower ones, when present in the same solution. Moreover, ninhydrin reacts also with some free amines, such as histamine. Over and above the presence of inorganic salts in any test solution will hinder the detection of protein bodies by ninhydrin. There are several other colorimetric, precipitating, salting, and dehydrating methods (Hawk *et al.*, 1954) for detecting the protein bodies, but the method as described above would help in detecting as well as estimating protein (or higher peptides) and lower peptides separately when present in the same solution in presence of other substances, such as polynucleotide and polysaccharide. By this colorimetric method, micro-quantities of protein and its degradation bodies can be separately spotted on paper and then eluted and estimated spectrophotometrically. Presence of any other organic matters and metallic salts does not significantly interfere with the reaction. Only a few drops (say 0.1 c.c.) of the test solution may be sufficient for complete analysis.

Beside the detection and estimation, this colorimetric method may be helpful in testing the homogeneity of a protein preparation. During fractionation of a protein preparation the ratio should have a constant value if the test sample be homogeneous, and in case of a heterogeneous preparation the ratio have variable values (*vide* Tables I to IV).

The mechanism of reaction between the dye (BPB) and the protein bodies has not yet been studied. However, it was noted that both free as well as the

calcium salt of the dye may react with protein bodies exactly in a similar way. In case of calcium salt, the linking ($-\text{SO}_2-\text{O}-$) in the molecule of the dye may play its rôle and the phenolic hydroxyl groups may be the linking point of the dye with the protein bodies.

An interesting phenomenon, observed during fractionation of egg white by paper ionophoresis, is that the different fractions showed different shades of colour, the more basic component producing deeper blue colour, while the more acidic one showing greenish colour. The colorimetric method may indicate the probable nature of the protein bodies.

The above described Method (I) detects and estimates protein and larger polypeptides (larger than proteoses), while Method (II) can detect and estimate polypeptides from proteoses downwards. By a combination of the two methods amount of proteoses present can be accounted for (*vide* Tables III and IV).

During the above investigational work, it was noted that the protein moiety of the nucleoprotein of guinea-pig liver showed a fairly uniform value for the ratio $\frac{\text{N}_2 \text{ mg./c.c.}}{\text{E}_{595} \text{ m}\mu}$ during fractionation with chloroform-octanol mixture (9:1).

Up-to-date literatures indicate that the highly polymerized nucleic acids are usually heterogeneous. In such cases the homogeneity of the protein moiety may be unexpected. Also, the fractionation of the protein with chloroform-octanol mixture may not be perfect. So, this point needs further investigation.

SUMMARY.

1. The ability of protein bodies to bind bromophenol blue was used to develop a simple method for evaluating protein components in various preparations. Native protein and higher peptides were analysed by using bromophenol blue with calcium chloride in presence of other protein degraded bodies as well as other macro-molecular bodies, such as polysaccharide and polynucleotide.

2. The lower peptides might be analysed again with mercuric chloride.

3. A combination of the two methods would estimate the protein or larger peptides content as well as its lower peptide contents separately.

4. Each protein component has its characteristic binding capacity for the dye and as such this colorimetric method is suitable to find out the degree of homogeneity of a protein preparation.

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THE THEORY OF THE ENDOGENOUS NITROGEN METABOLISM IN MAMMALS.

BY

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INTRODUCTION.

SINCE postulation of Folin's theory of protein metabolism (Folin, 1905) recognizing and differentiating the exogenous and endogenous types, considerable work has been done regarding their nature and origin. While the exogenous type was proved to be related to disposition of dietary proteins, controversy centred round the origin of the endogenous type. The endogenous protein metabolism was earlier supposed to represent a partial or complete disintegration of tissue proteins (Rubner, 1908; McCollum and Steenbock, 1912; Steenbock and Gross, 1918; Osborne and Mendel, 1914; Sherman, 1920; Cathcart, 1907 and 1921), but subsequent evidences (reviewed by Mitchell and Hamilton, 1929; Terroine and Sorg-Matter, 1927 and 1928; Brody *et al.*, 1934; Smuts, 1935; Burroughs *et al.*, 1940, *a*, *b* and *c*; Nielsen *et al.*, 1939, 1939*a*) offered indications of its close association with disintegration of non-protein nitrogenous tissue constituents. In the present paper, certain metabolic evidences have been presented to show that there existed a precise and concordant interrelationship among nitrogen, sulphur and phosphorus in fasting urine and in tissue proteins and a constant interrelationship of a different magnitude among them in endogenous urine and in non-protein tissue constituents of a nitrogen-free-fed mammalian organism. The identical nature of mutual interrelationships of these elements existing between the original biological matter in a living system and its catabolic end products in corresponding set of cases suggested that the endogenous nitrogen metabolism, while unrelated to the disintegration of tissue proteins of fasting metabolism, originated from and was associated with the catabolism of non-protein nitrogenous tissue constituents. These evidences further revealed that there was an endogenous metabolism of sulphur as well as that of phosphorus and the endogenous metabolism of these two elements was associated with and concomitant to the endogenous metabolism of nitrogen. An exponential equation has been developed to permit evaluation of the size of the non-protein nitrogen store in the body from the rate of endogenous nitrogen losses.

EXPERIMENTAL.

The experiments were conducted on four healthy human adults, whose ages varied between 18 and 28 years and body-weights between 45 and 60 kilos. The diet, which was constituted of 550 g. to 600 g. of sago and 150 g. to 200 g. of pure sugar per day with a little citrus juice, contained sufficient calories to satisfy energy requirements. Sago was an easily digestible starchy food free from any crude-fibre and sulphur and contained only 8 mg. of nitrogen and 5 mg. of phosphorus/100 g. of dry-stuff. Each experiment was conducted for a period of 6 to 7 days on an uniform intake of the experimental diet, following a short period of low-protein diet, and the excreta, voided only on the last 2 or 3 days, were quantitatively collected and analysed. Daily urine, which was collected and preserved by toluene to which some chloroform solution of thymol was previously added, was analysed for total N, total S and total P. Total N was estimated by the Kjeldahl method, total S by the Danis modification of Benedict method and total P by colorimetric method of Youngburn and Youngburn and the volumetric uranium-acetate titration method. Completeness of collection of urine was checked in every case by estimation of creatinine (Folin's method).

RESULTS.

Results of daily excretion of total nitrogen and sulphur in endogenous urine of the experimental subjects are recorded in Table I. Elimination of phosphorus, where analysed, was also noted therein along with the values of N/S and N/P ratios. Results of urinary elimination of these elements in the famous experiments of Smith (1926) and Duel *et al.* (1928) along with the N/S and N/P ratios are also given in Table I.

A highly significant positive correlation was found between nitrogen and sulphur eliminated in endogenous urine. The correlation coefficient between N and S was $+0.79$, significant at 0.1 per cent level. The nitrogen-sulphur relationship has been graphically represented in the Graph, by plotting S against N and a regression line has been fitted in. The values of N/S plotted against the corresponding values of N are found to lie on and close to the straight line running exactly parallel to the axis (N), indicating a constancy in the relative rate of elimination of nitrogen and sulphur. The average value of N/S was found to be 7.5 ± 0.20 . Similarly, there was a significant positive correlation between nitrogen and phosphorus eliminated in such endogenous urine. The correlation coefficient between nitrogen and phosphorus was $+0.57$. NP relationship has been graphically represented in the Graph. The average value of N/P ratio was found to be 7.2 ± 0.41 .

Results of intake and output of calcium and phosphorus in some of the aforesaid experiments are also obtained and given in Table II. From the metabolic balance sheet, it was observed that a calcium balance was established on an extremely low intake (82 mg.) of food calcium when the diet was practically free from phosphorus. On the other hand, on such a low calcium and phosphorus diet, the subjects were always on negative phosphorus balance and the average daily elimination of excess phosphorus over intake was of the order of 267 mg. While the average elimination of faecal calcium and faecal phosphorus was practically

similar, the elimination of excess phosphorus occurred by way of urine and this catabolic loss of excess phosphorus seemed to be unrelated to the metabolism of body calcium.

TABLE I.

Elimination of nitrogen, sulphur and phosphorus in endogenous human urine.

Reference.	Date.	Experi- mental subject.	Age and body- weight.	Total N per day (g.).	Total S per day (g.).	Total P per day (g.).	N:S.	N:P.
Smith (1926)	1-6-38	G.C.N.	18 years 49 kilos.	1.46	0.17	...	8.5	...
				1.93	0.22	...	8.7	...
				1.10	0.13	...	8.4	...
				1.50	...	0.19	...	7.8
	18-10-39	,,	19 years 50 kilos.	2.33	0.30	0.47	7.7	4.9
				2.12	0.28	0.39	7.5	5.4
	20-12-38	S.N.D.	24 years 49 kilos.	2.71	0.37	0.31	7.3	8.7
				2.17	0.32	0.26	6.7	8.3
				2.02	0.28	0.20	7.2	10.1
				1.94	0.31	0.24	6.2	8.0
	5-10-39	P.C.D.	22 years 45 kilos.	1.99	0.23	0.20	8.6	9.9
				1.62	0.16	0.23	10.1	7.0
Deuel <i>et al.</i> , (1928)	13-9-40	G.C.D.	23 years 60 kilos.	1.67	0.20	...	8.3	...
				1.54	0.25	...	6.1	...
	21-4-23	M.S.	26 years 64.7 kilos.	2.09	0.29	...	7.2	...
				2.01	0.29	...	6.9	...
				1.86	0.29	...	6.4	...
				1.68	0.23	...	7.3	...
	14-7-25 to 19-7-25 20-7-25 25-8-25	H.J.D.	73 kilos.	1.58	0.19	...	8.3	...
				2.32	0.31	0.26	7.4	8.9
				2.10	0.29	0.24	7.2	8.7
				2.32	0.28	0.46	8.2	6.0
				2.22	0.25	0.41	8.8	5.4
				2.58	0.28	0.33	9.2	7.8
1.90				0.26	0.23	7.3	8.2	
1.79				0.30	0.22	5.9	8.1	
							7.5±0.20	7.2±0.41

Correlation coefficient between N and S = +0.79 (significant at 0.1 per cent level).

Standard error = ±0.034.

Correlation coefficient between N and P = +0.57 (significant at 5 per cent level).

Standard error = ±0.077.

DISCUSSIONS.

Nitrogen-sulphur relation.—In fasting mammalian metabolism, the excretion of nitrogen and sulphur in urine exhibited a constant ratio of 15.2 ± 0.5 (Table III), while the N:S ratio of different mammalian tissue proteins, as obtained from the analytical results of Masters and McCance (1939), was computed to be 15.2 ± 1.1 . Similar values of the N:S ratio of mammalian tissue proteins were also found by other workers (Cathcart, 1921; Khan and Goodridge, 1926; Wilson, 1925; Hutchinson and Morris, 1936; Cuthbertson and Munro, 1929; Bailey, 1937; and Beach *et al.*, 1942). It was thus obvious that fasting protein metabolism was associated with the disintegration of tissue proteins and the view was generally accepted.

GRAPH.

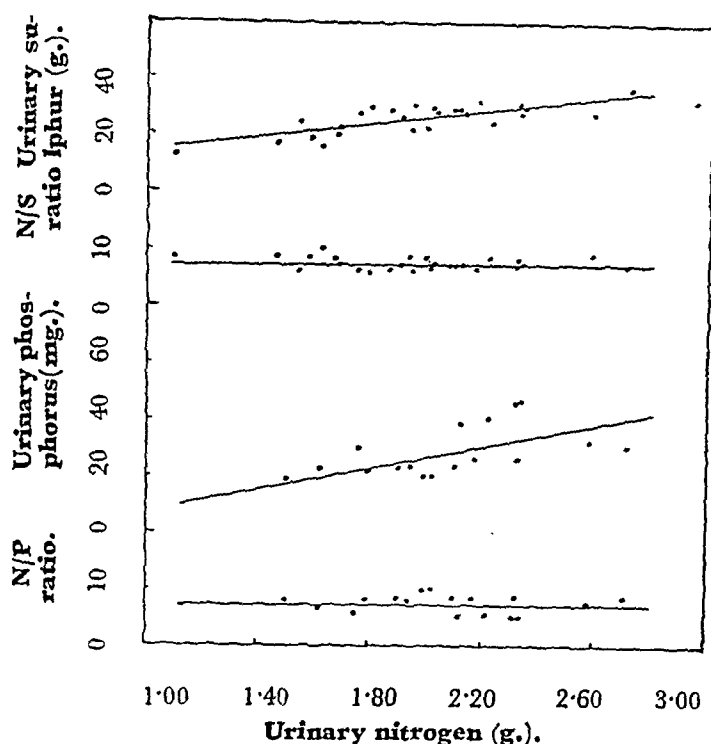


TABLE II.

Metabolic balance-sheet of galcium and phosphorus on N and P-free low Ca diets.

Date.	Subject.	Expt. day.	Dietary Ca.	Urinary Ca.	Faecal Ca.	Balance.	Dietary P.	Urinary P.	Faecal P.	Balance.
1-6-38	G.C.N.	Average	87	7	46	+34	39	192	45	-198
	G.C.N.	2nd	...	5	...	...	...	468	...	...
	"	3rd	...	5	...	...	...	387	...	...
	"	Average	82	5	44	+33	36	427	55	-446
20-12-38	S.N.D.	1st	...	22	...	...	...	311	...	...
	"	2nd	...	18	...	...	...	255	...	...
	"	3rd	...	8	...	...	...	196	...	...
	"	Average	77	16	58	+3	33	254	92	-313
5-10-39	P.C.D.	1st	...	35	...	...	...	239	...	...
	"	2nd	...	36	...	...	...	200	...	...
	"	3rd	...	38	...	...	...	227	...	...
	"	Average	82	36	83	-37	36	222	61	-247

Results are expressed in mg. on daily basis.

It was, however, found from the present studies that in the endogenous protein metabolism as per definition of Folin, which proceeded when the organism was provided with a complete diet except nitrogen (protein or amino acids), there was a highly significant positive correlation between urinary elimination of nitrogen and sulphur and the computed value of the N:S ratio was 7.5 ± 0.2 (Table I).

The difference in the N:S ratio of fasting and endogenous urine, therefore, indicated that the two types of metabolism were mutually different in nature and that the so called endogenous protein metabolism did not represent the disintegration of tissue proteins. On the other hand, the reconciliation of the N:S ratio of endogenous urine, as revealed in the present studies, with the N:S ratio of non-protein tissue extracts (7.04:1), as found by Mitchell, Nevans and Kendell (1922), showed that the endogenous nitrogen metabolism actually represented the disintegration of non-protein nitrogen and sulphur moiety of tissues.

TABLE III.

Nitrogen-sulphur ratio in fasting urine.

Reference.	Subject.	Expt. period, days.	Average N:S ratio.
<i>Human:</i>			
Khan and Goodridge (1926)	Cetti	6	14.7
" " " " ...	Breithaupt	5	15.1
Freunds (1901) " " ...	Succi	12	14.6
Cathcart (1907) " " ...	Beaute	11	15.5
Halpern (1908) " " ...		At the end of 20 days	14.6
Benedict (1915) " " ...	S.A.S.	Last 4 days	16.3
" " " " ...	L.	30	15.7
			<u>15.2±0.6</u>
<i>Ruminant:</i>			
Hutchinson <i>et al.</i> (1936) ...	Cow B	10	15.9
Morris <i>et al.</i> (1939) ...	Cow 1	12	14.6
		(except 1st day)	
" " " " ...	Cow 2	12	14.9
" " " " ...	Goat 1	Last day	14.4
" " " " ...	Goat 2	10	15.1
" " " " ...	Goat 3	12	15.3
" " " " ...	Goat 4	12	15.6
			<u>15.1±0.5</u>

Nitrogen-phosphorus relation.—The results in Table II will show that while the subjects were just in calcium balance even on a low intake of calcium (82 mg. per day), they always exhibited a noted deficit in phosphorus balance and the average deficit was about 267 mg.P per day on an average intake of about 36 mg.P. Elimination of faecal calcium and faecal phosphorus was practically similar (Ca:P=1:1.08). The excess elimination of urinary phosphorus, which seemed to be unrelated to the metabolism of calcium, was an indication of the endogenous phosphorus metabolism in the system.

It was also found that there was a significant positive correlation between nitrogen and phosphorus eliminations in endogenous urine and the computed value of the N:P ratio was 7.2 ± 0.41 (Table I). The nitrogen-phosphorus relationship suggested further examination as to whether tissue proteins constituted the materials for functioning of the endogenous phosphorus metabolism.

The N:P ratio of mammalian tissue proteins was found by Stearns (1939) as 15.3:1. It was also established from the results of numerous researches on men and animals (Benedict, 1915; Cathcart, 1921; Lusk, 1931) and no ruminants (Aylward and Blackwood, 1936; Morris and Ray, 1939) that in fasting metabolism, tissue proteins were virtually the sole catabolizing material and the N:P ratio of fasting urine and of tissue proteins was practically identical. Under the circumstances, the particular value of the N:P ratio of the endogenous urine would seem to be a characteristic manifestation of the endogenous phosphorus metabolism, and the wide difference in the value of this ratio and the N:P ratio of fasting urine would suggest that the endogenous phosphorus metabolism did not originate from and was not associated with the catabolism of tissue proteins.

The present studies also revealed that there was a constant mutual inter-relationship among the three nutritional elements in their endogenous metabolic functioning in the mammalian system such that the cellular catabolism of seven atoms of nitrogen of non-protein tissue constituents was concomitant to and associated with the cellular catabolism of one atom of sulphur and one atom of phosphorus from the same source.

Chemical nature.—Discussion about the chemical nature of the substances comprising the non-protein nitrogen moiety of tissues would seem premature due to inadequacy of information. There was, however, no doubt that it was a complex mixture of multiple chemical compounds inasmuch as forty known ninhydrin-positive compounds have been detected and most of them estimated chromatographically from the protein-free extracts of mammalian tissues (Tallan, Moore and Stein, 1954), besides a large number of other phosphorus and sulphur containing non-protein nitrogenous substances, whose physiological properties and metabolic functions were already known.

Evaluation of non-protein nitrogen store in the body from the rate of urinary nitrogen losses during nitrogen-free feeding.—As a result of normal functioning of the life processes, a quantum of nitrogen used in the endogenous nitrogen metabolism is lost from the non-protein nitrogen store of the body. If the loss is replenished from any exogenous sources of nitrogen or nitrogen released from dislodgment of tissue proteins, the endogenous nitrogen metabolism is presumably maintained at a constant level, as was hitherto believed. If the loss is not replenished, the non-protein nitrogen store is gradually diminished in proportion to the quantum of nitrogen successively lost through endogenous urine and such diminution of the store is followed by progressive reduction of the size of the successive quanta of nitrogen losses and of the overall rate of endogenous nitrogen metabolism.

An exponential curve $n = Ae^{\lambda t}$ was fitted where 'n' was the endogenous nitrogen output and 't' was the date of observation during the progress of nitrogen-free feeding. The parameter λ was negative and its value was -0.0381 in the 1st experiment (Smith, *loc. cit.*) and -0.0627 in the 2nd experiment (Deuel *et al.*, *loc. cit.*); the value of A was calculated as 2.4690 and 2.7367 in the respective cases. The theoretical values of 'n', as calculated from the curve, are shown in the last column of Table IV. The calculated values fairly agreed with the observed values, thus indicating the validity of the assumptions underlying the equation.

TABLE IV.

Calculated values of n from the equation $n = Ae^{\lambda t}$.

Reference.	Date of experiment.	RATE OF ELIMINATION OF ENDOGENOUS NITROGEN (MG. PER DAY):	
		Observed.	Expected.
Smith (1926)	April 16 (11th day)	2.37	2.38
	17	2.18	2.29
	18	2.10	2.20
	19	2.13	2.12
	20	2.16	2.04
	21	2.09	1.96
	22	2.01	1.89
	23	1.86	1.82
	24	1.68	1.75
	25 (20th day)	1.58	1.69
	August 24 (24th day)	2.51	2.57
	25	2.32	2.41
Deuel <i>et al.</i> (1928) Period 5.	26	2.22	2.27
	27	2.58	2.13
	28	1.90	2.00
	29	1.79	1.88
	30 (30th day)	1.75	1.76

From the aforesaid equation, it was possible to have an estimate of the non-protein nitrogen store of the body. The store on the 11th day of the experiment (corresponding to April 16) on Smith on nitrogen-free feeding, when the effect of the pre-experimental protein diet was presumed to have been completely eliminated and the functioning of the endogenous nitrogen metabolism alone prevailed, was calculated as 63 g. nitrogen. The store on the 24th day of the experiment on Deuel corresponding to August 24 worked out to 41 g. Extrapolating the values of endogenous nitrogen output for the 1st day of the experiment, the size of the store on that day worked out to be 91 g. (Smith) and 99 g. (Deuel), respectively. Hence, on the basis of the total nitrogen content of the adult human body as 2.304 per cent N (Mitchell *et al.*, 1945; and Widdowson *et al.*, 1951), the non-protein nitrogen store represented 6.11 per cent and 5.89 per cent of the total body nitrogen of the respective subjects under normal feeding.

SUMMARY.

1. Urinary elimination of total nitrogen, sulphur and phosphorus of 4 human adults of different ages and body-weights and maintained on adequate quantity of nitrogen-sulphur-phosphorus-free diets was determined. Significant positive correlation was found between nitrogen and sulphur and between nitrogen and phosphorus in endogenous urine. The average value of N:S and N:P ratios was calculated as 7.5 ± 0.20 and 7.2 ± 0.41 respectively.

2. The difference between the N:S ratio of endogenous urine and of fasting urine (and tissue-proteins) and the reconciliation of the former with the N:S

ratio of non-protein tissue constituents showed that the endogenous nitrogen metabolism did not represent the disintegration of tissue proteins but it was associated with and originated from the catabolism of non-protein nitrogenous tissue constituents.

3. Similarly, the N:P ratio in endogenous urine of NP-free-fed human adults, under metabolic conditions giving no indication of demineralization of osseous tissues, suggested that the endogenous metabolism of phosphorus represented the catabolism not of tissue proteins but of certain non-protein nitrogenous tissue constituents, which were relatively richer in phosphorus than tissue proteins.

4. The endogenous nitrogen metabolism seemed to be interrelated to the endogenous metabolism of sulphur and phosphorus such that the cellular catabolism of 7 atoms of nitrogen of non-protein tissue constituents was concomitant to the cellular catabolism of 1 atom of sulphur and 1 atom of phosphorus from the same source.

5. An exponential equation was developed to incorporate the relation between the size of the non-protein nitrogen store in the body and the rate of urinary nitrogen losses during the course of continued nitrogen-free feeding. It was estimated that the non-protein nitrogen store represented about 6 per cent of the total nitrogen store in human adults on normal feeding.

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EFFECT OF PROTEIN SUPPLEMENTATION AND SOME SO-CALLED 'GALACTOGOGUES' ON LACTATION OF POOR INDIAN WOMEN.

BY

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ONE of the most gratifying features with regard to the nutritional situation of poor Indian communities, is the remarkable ability of the women to breast-feed their infants successfully over prolonged periods. In a field study of the lactation performance of women of different socio-economic and cultural groups in the Nilgiris District of South India, it has been found that inability to breast-feed for a minimum period of six months, which was a frequent finding among the high socio-economic group, was practically unknown among the poor (Gopalan, 1957). A longitudinal study of a group of poor Indian women revealed that lactation in these women was maintained at a satisfactory level well beyond the end of the first year (Gopalan, 1957). The composition of the breast milk of the poor mothers was found to correspond to that of European mothers, at least with regard to some of the proximate principles (Srinivasan and Ramanathan, 1954).

This satisfactory situation with regard to lactation, obtaining among the poor communities, must be considered a most valuable asset, to be preserved, protected and, if possible, augmented. Surveys of the diets of these poor mothers reveal all-round inadequacy. For example, in a group of poor mothers in different stages of lactation ranging from one week after delivery to two years after delivery, the calorie intake was found to range roughly between 1,400 and 2,400, the protein intake between 45 g. and 60 g. daily and the milk output in 24 hours between 12 oz. and 21 oz. (Gopalan, 1955). A satisfactory lactation performance in the face of such inadequate diet could only be achieved through tissue depletion. Nitrogen balance studies during lactation, in a group of poor Indian mothers

actually revealed increasing N retention with increasing protein intake (Narasinga Rao *et al.*, 1958). Under the circumstances, the question has to be considered whether an increased protein intake in the dietaries of these mothers would be reflected in an improved output of breast milk. This question has immense practical implications. If protein supplementation to the mother improves her lactation performance and thus indirectly improves the nutrition of the infant, it would be easier, more practicable and rational, to achieve the nutritional improvement of the infants in this manner in the first few months after birth than through direct supplementation to the infant.

Among the poor communities, several articles of diet and some drugs are reputed to possess galactogogic properties. These substances are actually administered to the mother, immediately after delivery to promote lactation and are sometimes continued for sometime thereafter with a view to maintain lactation. Precise proof of the galactogogic properties of these substances is, however, not available.

In the present investigation, the effect of protein supplementation on the lactation of poor mothers has been investigated. An attempt has also been made to study the effect of certain substances which are reputed, among these poor communities, to promote lactation.

MATERIALS AND METHODS.

(A) *Effect of protein supplementation.*—This investigation was carried out on the following two lines:

1. A group of six mothers belonging to the poor socio-economic class and in various stages of lactation (5 to 13 months) were admitted to the hospital. They were maintained for ten days on a diet supplying about 2,900 calories and 61 g. protein. In the last 3 days of this 10-day period, test-feeding of the infants was carried out with a view to determining the total amount of milk consumed by the infant in each 24 hour period. The infants were weighed in an accurate lever-actuated balance before and after every feed and the total amount of milk consumed in 24 hours calculated. Samples of the milk obtained on each of these 3 days were analysed for nitrogen. For the next ten days, the mothers were placed on a diet supplying 99 g. of protein; but the calorie intake was maintained at the same level as before. In the last three days of this ten-day period, test feeding of the infants was again carried out and samples of milk analysed for nitrogen. For the next ten days, the protein level in the diet was raised to 114 g., keeping the calorie level constant. In the last 3 days of this ten-day period also, test-feeding of infants was again carried out and samples of milk analysed for nitrogen. Throughout the investigation, it was ensured that the mothers consumed only the diet administered to them and that completely.

2. Thirty mothers belonging to the poor socio-economic class were divided into two groups of 15 each, which were comparable with regard to age composition, parity and occupational and environmental status. The mothers and their

infants were taken up for observation immediately after delivery. All mothers were allowed to live in their own homes, on their usual diets and performing their usual occupations. Diet surveys did not reveal any significant differences with regard to the intake of different nutrients between the members of the two groups. The average protein intake in the dietaries was of the order of 60 g. daily.

Mothers of one group were given a daily supplement of 3 oz. of skim milk powder supplying 30 g. of protein. The milk was actually delivered at the homes and it was ensured that the skim milk was reconstituted and consumed by the mothers in the presence of the attending nurse. Mothers of the second group were given a placebo. All mothers were instructed to continue their usual diets; mothers of the skim milk supplemented group were specially advised not to eliminate any item of their usual food because of the supplement they were getting.

Mothers and their infants were weighed on a sensitive lever-actuated balance once a fortnight. The investigation continued for six months after delivery during which period it was ensured that none of the infants received any supplements and that all infants were solely breast-fed. At the end of the six months, serum proteins, hæmoglobin and serum calcium were estimated in all the mothers.

(B) *Effect of so-called galactogogues.*—The substances claimed to possess galactogogic properties, investigated in the present study were: (i) Garlic (*Allium sativum*), (ii) cotton seed (*Gossypium hirsutum*), (iii) tamarind (*Tamarindus indica*), and (iv) 'Leptaden' (an indigenous preparation consisting of 2 ingredients 'Jeevanti' and 'Kamboji' claimed to possess powerful galactogogic properties).

The procedure employed to investigate the effects of these substances on lactation was as follows: Mothers in different stages of lactation were admitted to hospital. Test-feeding of their infants was carried out for 72 hours continuously and the average output of milk in 24 hours for each mother determined. The mothers were then discharged. The mothers were now divided into four groups; mothers of each group were administered one of the four substances under investigation daily for periods ranging from 15 days to one month in their own homes. At the end of this period, they were again admitted to the hospital and test-feeding of their infants carried out for 72 hours continuously to assess the average 24 hours output of milk by each mother.

The substances were administered to the lactating mothers in the forms in which they were usually consumed by them for this purpose. Half an ounce of garlic, crushed, fried and cooked with little salt and water, sweetened and flavoured was administered daily to each patient of one group. Four ounces of cotton seed were soaked in water for about two hours, ground and the milk extracted and made up to 24 oz.; 4 oz. of this milk were fed daily to each subject of the second group. Tamarind juice was extracted by squeezing one- and-half oz. of tamarind in 16 oz. of water; 4 oz. of this fluid were administered daily to each patient of the third group. Three tablets of 'Leptaden' daily were administered to each patient of the fourth group.

320 Protein Supplementation and 'Galactogogues' of Poor Indian Women.

RESULTS.

The results of the above investigations are set out in Tables I, II, III and the Graph:—

TABLE I.

Milk yield in 24 hours and protein content† of milk at different levels of protein intake.*

Case number.	I PERIOD (61 G. PROTEIN):			II PERIOD (99 G. PROTEIN):			III PERIOD (114 G. PROTEIN):		
	Milk yield, c.c.	Protein g./100, c.c.	Total protein in 24 hours milk.	Milk yield, c.c.	Protein g./100, c.c.	Total protein in 24 hours milk.	Milk yield, c.c.	Protein g./100, c.c.	Total protein in 24 hours milk.
1	502	1.16	5.8	639	0.96	6.1	562	0.97	5.4
2	287	1.54	4.4	434	1.29	5.6	423	1.25	5.3
3	318	0.88	2.8	434	0.80	3.5	443	0.89	3.9
4	459	1.36	6.4	456	1.17	5.3	515	1.10	5.7
5	430	1.26	5.4	536	0.88	4.7	499	1.95	4.7
6	417	1.06	4.4	589	1.01	5.9	496	1.03	5.1

* and † Figures in=table represent the average of three days measurements.

TABLE II.

Comparison of the growth of infants in the supplemented and control group.

Group.	AVERAGE INCREASE OF BODY-WEIGHT OF INFANTS IN OZ. EVERY FORTNIGHT FOR SIX MONTHS:												
	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th	Total
Milk supplemented (15 cases) ...	13.6	14.2	13.4	14.0	11.5	12.2	10.3	8.3	8.5	6.5	7.2	5.2	124.9
Control (15 cases) ...	11.2	14.1	13.6	12.8	10.0	9.9	9.0	9.1	10.9	6.8	7.1	5.8	120.3

*Average birth weight of the milk-supplemented group 6 lb.-1/2 oz.; of the control—6 oz.

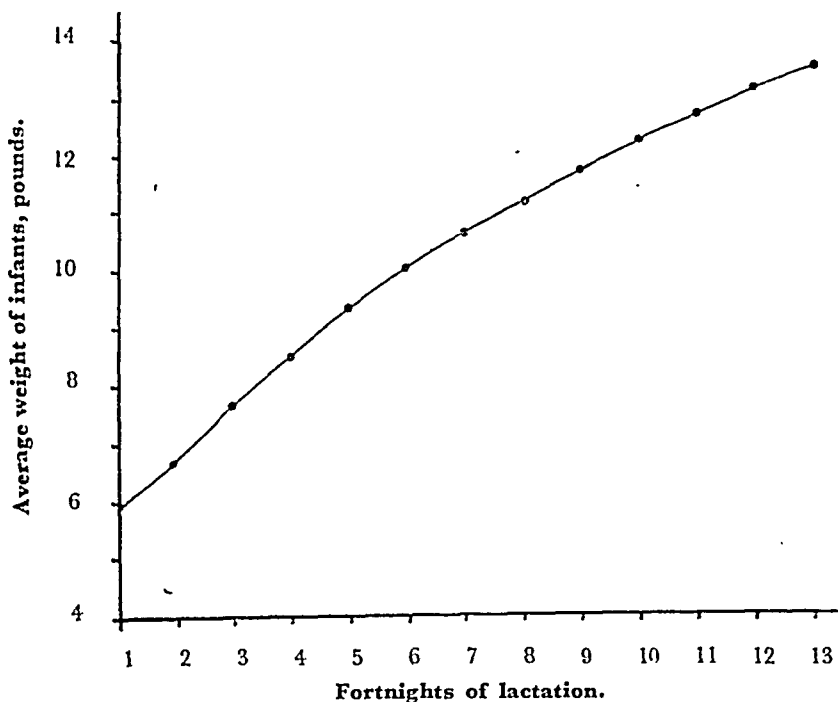
TABLE III.

Effect of different so-called galactogogues on lactation.

LEPTADEN:				COTTON SEED:				TAMARIND:			GARLIC:		
Case number.	Starting milk yield, c.c.	Milk yield after 7 days, c.c.	Milk yield after 14 days, c.c.	Case number.	Starting milk yield, c.c.	Milk yield after 2 days, c.c.	Milk yield after 14 days, c.c.	Case number.	Starting milk yield, c.c.	Milk yield after 3 weeks, c.c.	Case number.	Starting milk yield, c.c.	Milk yield after 14 days, c.c.
1	568.0	568.0	540	6	426.0	366	319.5	12	506	639	16	379	458
2	454.0	468.6	561	7	511.2	451	511.2	13	535	511	17	459	451
3	568.0	459.1	469	8	546.7	394	497.0	14	660	447	18	518	600
4	653.0	568.0	405	9	504.1	472	418.9	15	419	511	19	447	430
5	426.0	454.4	440	10	518.3	437	518.3				20	497	394
::				11	433.1	391	468.6				21	296	327*
											22	315	440*

* Garlic given for 30 days.

GRAPH.



It will be seen from Table I that an increase in protein intake from 61 g. to 99 g. daily resulted in an appreciable increase in the 24 hour output of breast milk in five of the six patients investigated. A further increase of protein intake to 114 g. daily had no beneficial effect. It was interesting, however, that the increase in the output of milk following on the increase in protein intake of the mother from 61 g. to 99 g. daily, was attended with a diminution in the percentage of protein in the milk with the result that the overall increase in total protein output in milk in the 24 hours period was not as appreciable as the increase in the quantity of milk.

Comparison of the growth of infants in the milk-supplemented and control groups (Table II) did not reveal any striking difference. Serum protein, hæmoglobin and serum calcium estimations carried out in all the mothers at the end of 6 months did not show any significant differences. There was no significant difference with regard to the changes in body weight of the mothers in the two groups.

It will be seen from Table III that none of the so-called 'galactogogues' investigated here had a significant effect on the output of breast milk. However, 5 of the 10 subjects investigated with garlic, reported without specific questioning, a subjective feeling of breast engorgement and improved milk output.

The investigation, incidentally, provided an opportunity to follow the growth performance of a group of 30 infants from birth to the end of six months. These

3. None of the so-called galactogogues investigated was found to have a significant effect on lactation; under the conditions obtaining in the investigation.

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NITROGEN BALANCE STUDIES IN POOR INDIAN WOMEN DURING LACTATION.

BY

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DURING pregnancy and lactation, considerable additional demands are imposed on the maternal organism and the nutritional requirements of the mother are greatly enhanced. There is a considerable body of epidemiological, experimental and clinical evidence, pointing to the increased nutritional demands of pregnancy and lactation. However, information about the precise nutritional requirements in pregnancy and lactation is lacking.

Coming to the specific question of protein requirements in pregnancy and lactation, the Nutrition Advisory Committee of the Indian Council of Medical Research (1944) had recommended an allowance of 101 g. protein daily during pregnancy and 112 g. protein daily during lactation for a woman weighing about 45 kg. There are similar recommendations from the nutritional organizations of other countries. It would, however, appear that these recommendations are not based on any exact scientific data.

All available evidence would indicate that the nutritional requirement in pregnancy is not merely the nutritional requirement of the mother in her normal state plus the nutritional requirement of the foetus. Similarly, the nutritional requirement of the lactating mother is not evidently just the requirement of the woman in her normal state plus the amount represented by the nutrients in the breast milk. Presumably, there are profound alterations in metabolic behaviour during pregnancy and lactation, which have not been fully understood. The information available on this subject, with regard to the lactating woman is even scantier than that with regard to the pregnant woman.

Nitrogen balance studies in pregnancy (Hunscher *et al.*, 1933; Macy and Hunscher, 1934; Coons and Marshall, 1934; Coons *et al.*, 1935; Hunscher *et al.*, 1935) have shown that women store relatively large amounts of protein, during pregnancy far and above the amounts needed by the foetus and accessory structures. There would, however, appear to be a substantial loss of nitrogen from the maternal organism during parturition and the postpartum period (Macy and Hunscher, *loc. cit.*). The additional loss in the breast milk has also to be reckoned with during lactation. The only available data on nitrogen balance studies in lactation are those of Macy and coworkers (Macy *et al.*, 1930; Hunscher *et al.*, 1933; Macy and Hunscher, *loc. cit.*). These workers had carried out nitrogen balance studies in presumably well-nourished women during lactation at intakes of 25 g. to 27 g. N per day and found retention of the order of 1.0 g. per day.

It might be expected that the protein requirement in lactation would be determined by the protein intake of the mother during her normal state, during the entire period of her pregnancy, and her current lactation performance. To be able to assess the precise physiological protein requirement in lactation, it would be necessary to investigate normal women who have had optimal protein intake during their pregnancy, and with a normal lactation performance.

In the present study, however, nitrogen balance determinations at three levels of protein intake have been carried out on a group of Indian women belonging to the poor socio-economic group. It is believed that the data collected here may provide information regarding the actual protein requirements of the women *under their prevalent conditions* and also provide some indirect clue regarding the physiological protein requirement in lactation.

MATERIALS AND METHODS.

Six apparently normal women in different stages of lactation (five to thirteen months) belonging to the low socio-economic group were selected for the study. The relevant data about these subjects including their dietary history are given in Table I. They were kept in the hospital during the entire period of

TABLE I.

Data regarding subjects investigated.

Subject number.	Age in years.	Lactating period, months.	Weight, kg.	DIETARY HISTORY:			
				Calories.	Protein, g.	Carbo-hydrate, g.	Fat, g.
1	26	6	41.8	...	...	...	...
2	30	9	33.6	...	...	...	...
3	38	6	34.1	2,733	70	548	19
4	26	6	38.2	2,402	63	474	24
5	28	5	39.1	2,233	50	473	7
6	32	13	34.1	2,440	61	482	24

metabolic study which lasted 30 days, to enable strict supervision. Nitrogen balance study was carried out at three levels of protein intake, but the calorie intake was the same throughout. The duration of study with each of the three protein levels was 10 days. The first seven days of this 10-day period were allowed for the subjects to stabilize on the diet of the particular period; urine and faeces were collected and the output of nitrogen in breast milk assessed during the last three days. The weights of the subjects were recorded at the beginning and at the end of each diet period and during the three days collection period.

Diets.—Strict care was maintained over the preparation and feeding of the diets. The composition of the diet fed to these subjects is given in Table II. The protein level of the diet in the first period was not much higher than the usual

TABLE II.

Composition of the diet of the subjects.

Components.	First period.	Second period.	Third period.
Protein g. ...	61	99	114
Calories ...	2,938	2,991	2,999
Fat, g. ...	54	54	48

protein intake of these subjects in their home diets. The articles of the diet were accurately weighed and cooked in the laboratory. It was ensured that the subjects consumed all the diets supplied to them.

Urine.—Twenty-four hour output of urine was collected over 5 c.c. of toluene and analysed for total nitrogen by micro-Kjeldahl procedure. Creatinine was estimated in the urine by the method of Clark and Thompson (1949) as a check over completeness of collection. Precautions were taken to ensure complete collection of all urine passed in the twenty-four hours.

Faeces.—Faeces passed during twenty-four hours was thoroughly mixed and a representative sample (*Ca.* 50 g.) was taken and dried with a little oxalic acid to a constant weight in a hot-air-drier. The dried faeces was powdered and nitrogen determined in the sample by macro-Kjeldahl procedure. Strict precautions were taken to ensure complete collection of faeces in each twenty-four hour period.

Milk.—In order to find out the total daily output of milk these subjects, test feeding of their infants was carried out during the three days collection period. A sample of milk, each day, was analysed for nitrogen by micro-Kjeldahl method.

RESULTS.

The results have been set out in Tables III and IV and the Graph.

It is seen from Table III that on an intake of 61 g. of protein daily the subjects were in the neighbourhood of nitrogen balance. However, when the protein intake was raised to 99 g. per day considerable positive nitrogen balance

was induced in all the subjects. This positive balance was either maintained or slightly increased when the daily protein intake was raised to 114 g. (in one case there was slight fall in retention which could be attributed to increase in faecal nitrogen). In all cases the faecal nitrogen was higher than usual. This could be attributed to relatively incomplete absorption temporarily following on the change-over from home diet to the experimental diet in these subjects.

TABLE III.

Nitrogen balance in mothers during their lactation on three different levels of protein intake.

Subjects.	I PERIOD N INTAKE=9.81 g:					II PERIOD N INTAKE=15.88 g:					III PERIOD N INTAKE=18.09 g:				
	Nitrogen in g.					Nitrogen in g.					Nitrogen in g.				
	Faecal.	Urinary.	Milk.	Total excretion.	Balance.	Faecal.	Urinary.	Milk.	Total excretion.	Balance.	Faecal.	Urinary.	Milk.	Total excretion.	Balance.
1	3.75	4.56	0.89	9.20	+0.61	3.62	7.35	0.98	11.95	+3.93	4.29	8.36	0.90	13.55	+4.54
2	2.90	6.00	0.71	9.61	+0.20	5.41	7.85	0.96	14.32	+1.56	3.88	9.32	0.85	14.05	+4.05
3	4.10	5.01	0.46	9.57	+0.24	2.64	6.86	0.59	10.09	+5.79	3.79	7.90	0.63	12.32	+5.77
4	2.92	7.12	0.96	11.00	-1.19	4.73	7.05	0.85	12.63	+3.25	3.95	9.18	0.90	14.03	+4.06
5	3.79	4.87	0.80	9.46	+0.35	3.94	5.58	0.75	10.27	+5.61	4.91	7.49	0.76	13.16	+4.93
6	2.89	5.00	0.66	8.55	+1.26	3.06	7.70	0.95	11.71	+4.17	2.66	8.82	0.82	12.30	+5.79

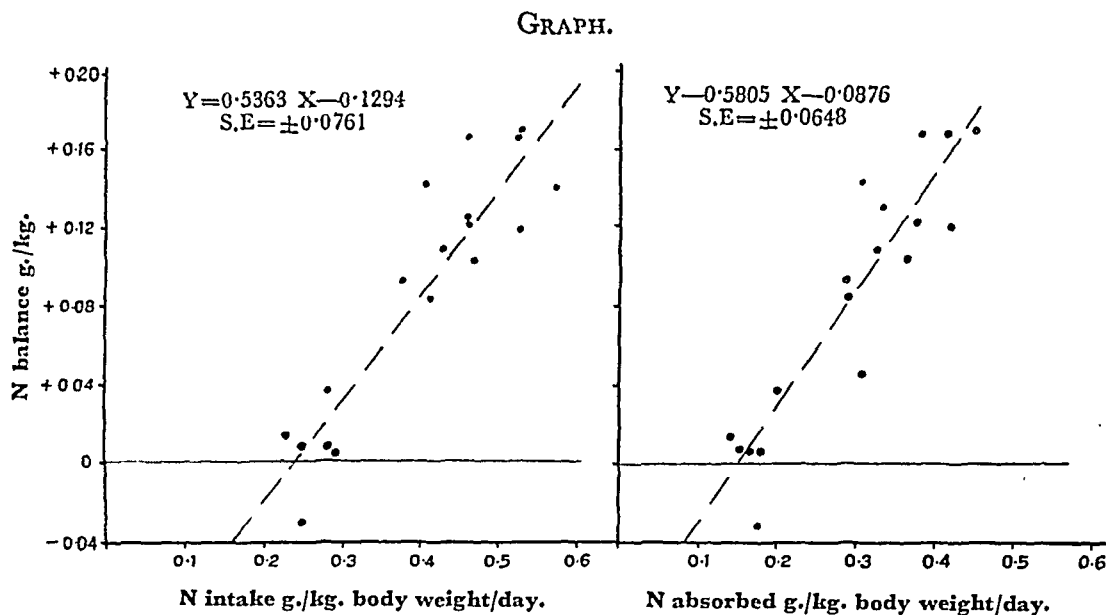
(Figures represent average of three 24-hour collections).

TABLE IV.

Relationship between nitrogen retention and weight changes.

1ST PERIOD:		II ND PERIOD:		III RD PERIOD:	
Weight change during 10 days, kg.	N retention per day, g.	Weight change during 10 days, kg.	N retention per day, g.	Weight change during 10 days, kg.	N retention per day, g.
0	-1.19	0	1.56	0	4.06
+1.36	+0.35	0	5.61	0	4.54
+1.82	+1.26	+0.46	3.93	+0.91	4.05
+2.27	+0.20	+0.91	5.79	+0.91	5.77
+2.73	+0.24	+1.36	4.17	+0.91	5.79
+4.55	+0.61	+2.27	3.25	+2.27	4.93

In the Graph, nitrogen retention has been plotted against nitrogen intake and nitrogen absorbed. There was a linear relationship between nitrogen intake and N retention ($Y=0.5363X-0.1294$). There was an even more striking relationship between N absorbed and N retained ($Y=0.5805X-0.0876$). In



Relationship of N balance to N intake and N absorbed,

both cases the regression coefficient is significant. Wider scatter in the former was due to variation in the faecal nitrogen from one period to another period. From these graphs it would appear that 0.24 g. nitrogen per kg. body-weight per day would be the minimum nitrogen intake required for maintaining nitrogen equilibrium in these subjects. This value is higher than that reported by Phansalkar and Patwardhan (1956) for normal Indian adults (38.2 g. protein for an adult weighing 70 kg. or 0.087 g. N per kg. body-weight per day). Even if allowance is made for higher faecal excretion in these subjects, and the minimum nitrogen absorbed required for maintenance of N equilibrium is considered, these subjects required 0.15 g. N per kg. body-weight per day as against 0.087 g. N per kg. body-weight per day required by the normal subjects investigated by Phansalkar and Patwardhan (*loc. cit.*).

It can be seen from Table IV that weight changes in these subjects were no reflection of nitrogen retention, a feature observed repeatedly in nitrogen balance studies. Though average nitrogen retention was minimal (0.25 g. per day) during the first ten days, average weight gain (2.12 kg.) was maximal during this period. On the other hand, during the second and third periods, though the average retention of nitrogen was 4.05 g. and 4.86 g. per day respectively, average weight gain was only 0.83 kg. during each period.

DISCUSSION.

The lactating women studied here were in nitrogen balance on an intake of 0.24 g. per kg. body-weight per day. This is much above the reported minimum nitrogen of 0.087 g. per kg. body-weight per day required for nitrogen balance in normal Indian adults (Phansalkar and Patwardhan, *loc. cit.*). Even taking

into consideration that the subjects excreted fairly large amounts of nitrogen in faeces (3.4 g. daily compared to 1.5 g. to 2.0 g. in normals), the value for minimum protein for maintaining balance in these subjects was certainly raised. The present observation, thus, justifies the need for larger quantity of protein during lactation than for normal adults, a concept which has hitherto been based mainly on computations of nitrogen secretion in milk. Thus, the minimum protein needs during lactation must be considered to be above 1.5 g. protein per day per kg. body-weight, and nearly three times the minimal requirement of normal subjects.

The subjects studied here, though apparently normal, were drawn from the poorest segments of the population. They had gone through their pregnancies without any special improvement of their usual diets. It might be expected, therefore, that they all suffered from varying degrees of relative tissue protein depletion. In interpreting the results of the metabolic studies on these subjects, the possible effects of the superimposition of the background of malnutrition on their lactation state must be considered. The work of Allison (1949) has shown that in states of protein depletion, the minimum level of nitrogen needed to attain nitrogen balance is lowered. This would lead one to conclude that the minimum level of nitrogen required to attain nitrogen balance in the lactating subjects investigated here would have been even higher if their erstwhile protein nutritional status was excellent. It would follow from this that the physiological requirement of protein in lactation is probably even higher than the level of 1.5 g. protein daily per kg. body-weight, arrived at in the present investigation. For example, Macy *et al.* (*loc. cit.*) and Hunscher *et al.* (1933) who carried out N balance studies at a level of 25 g. N per day obtained a positive balance of only about 1 g. per day.

In the subjects investigated here, apart from elevation of the minimum level of nitrogen needed for the attainment of balance discussed above, it was found that nitrogen retention increased as the protein intake was raised. This latter phenomenon might not be a specific attribute of the lactation state but could be the result of relative tissue protein depletion of the subjects investigated here. In support of this may be quoted the work of Beattie *et al.* (1947) and Benditt *et al.* (1948) who had shown that in undernutrition there was a linear relationship between nitrogen retention and nitrogen intake. In computing the actual protein needs of the malnourished lactating women investigated here, the protein level which results in maximal nitrogen retention should be considered the optimal requirement. In the present study an increase of intake to 99 g. per day from 61 g. protein per day resulted in increased retention, but when the intake was raised to 114 g. from 99 g., there was no appreciable further increase in retention. Using this criterion, an intake of protein 100 g., or above derived from the type of diets which have been studied per day is obviously needed by these mothers to meet the increased demand of lactation and to discharge their tissue protein deficit.

SUMMARY.

1. Nitrogen balance study was carried out at three levels of protein intake on six Indian women belonging to poor socio-economic group during lactation.

2. These subjects were in the neighbourhood of N balance on an intake of 61 g. protein per day. By increasing the daily protein intake to 99 g., considerable positive nitrogen was induced in all the subjects. Further raising the intake to 114 g. had little additional effect.

3. Based on this data it would appear that minimum protein required for nitrogen balance in lactating woman is above 1.5 g. per kg. per day. Considering the protein requirement as that which results in maximal nitrogen retention, malnourished lactating women studied here require 100 g. or above of protein per day derived from the type of diets employed in the present investigation.

The authors wish to thank Dr. A. D. Taskar for statistical analysis of the results.

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NUTRITIVE VALUE OF VEGETABLE PROTEINS.

Part II.

THE EFFECT OF VEGETABLE PROTEIN DIETS ON THE REGENERATION OF HÆMOGLOBIN AND PLASMA PROTEINS IN PROTEIN-DEPLETED RATS.

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INTRODUCTION.

SEVERAL investigators have demonstrated that the regeneration of plasma proteins (Kerr, Hurwitz and Whipple, 1918; Madden and Whipple, 1940; Allison, Anderson and White, 1949; Chow, Seeley, Allison and Cole, 1948) and of hæmoglobin (Holman, Mahoney and Whipple, 1934; Orten and Orten, 1943 and 1945; Damodaran and Vijayaraghavan, 1943; Vijayaraghavan, 1955) in protein-depleted animals is largely influenced by the quality of dietary protein. Whipple and Robschiet-Robbins (1951) have reported that dietary proteins differ very much in their capacity to regenerate plasma protein and hæmoglobin respectively as they do in their capacity to support growth or maintain nitrogen equilibrium. In view of this, it becomes necessary in any investigation on the determination of the nutritive value of proteins to determine, besides their biological value for growth and maintenance, their efficiency in the regeneration of hæmoglobin and plasma protein. In an attempt to evolve suitable combinations of dietary vegetable proteins for the prevention and treatment of protein malnutrition, Phansalkar, Ramachandran and Patwardhan (1957) studied the protein efficiency ratios (P.E.R.) in albino rats of mixtures of cereal, pulse and leafy

vegetable proteins. They observed that the P.E.R. of the different cereal-pulse-vegetable combinations was significantly higher than those of the individual constituents and further that it compared favourably with the P.E.R. of skim milk. Phansalkar and Patwardhan (1956) had earlier observed that supplementation of cereals with pulses and leafy vegetables improved the biological value and egg replacement value of the mixed protein in adult human subjects. In view of these encouraging results, it was felt necessary to compare with skim milk protein the capacity of these mixed vegetable proteins to regenerate hæmoglobin and plasma proteins in protein depleted animals. The present communication is an account of the observations made in adult albino rats of the changes in hæmoglobin and plasma proteins in the process of protein depletion followed by repletion.

EXPERIMENTAL AND RESULTS.

Different methods have been used in the past for the production of anæmia and hypoproteinemia in experimental animals. Whipple and Robscheit-Robbins (1940) have accomplished this in dogs by periodic bleeding of the animals maintained on protein-free diets. Orten and Orten (*loc. cit.*) have shown that hæmoglobin level fell to 11.5 g. per cent in rats by feeding protein-free diet for a prolonged period. Aschkenasy (1957) has also produced anæmia in rats with diminished hæmoglobin concentration and erythrocyte count by feeding a protein-free diet. Chow, Allison, Cole and Seeley (1945) have brought about reduction in total circulating plasma proteins in dogs either by feeding protein-free diet only or in conjunction with plasmapheresis.

According to Whipple, Miller and Robscheit-Robbins (1947), the synthesis of hæmoglobin and plasma protein occurring at the cost of tissue protein must interfere in the evaluation of the effect of dietary protein on regeneration of these constituents unless the depletion of body stores of protein is first achieved. In view of this it was considered necessary in the present experiment not merely to deplete the animals of their body proteins but also to determine the degree of protein depletion resulting from protein-free diets in order to be able to decide when the repletion should start. It has been shown by several workers (Metcoff, Favour and Stare, 1945; Allison, 1948; Allington and Taylor, 1955; Hope and Verel, 1955; Wintrobe, 1954; Hallgren, 1953) that the concentrations of hæmoglobin and plasma proteins alone do not give as correct an indication of the degree of depletion of blood proteins as the information on the total circulating amounts of these constituents. Hence, blood and plasma volume estimations were made together with the determination of hæmoglobin and total plasma protein concentration during depletion and also during repletion.

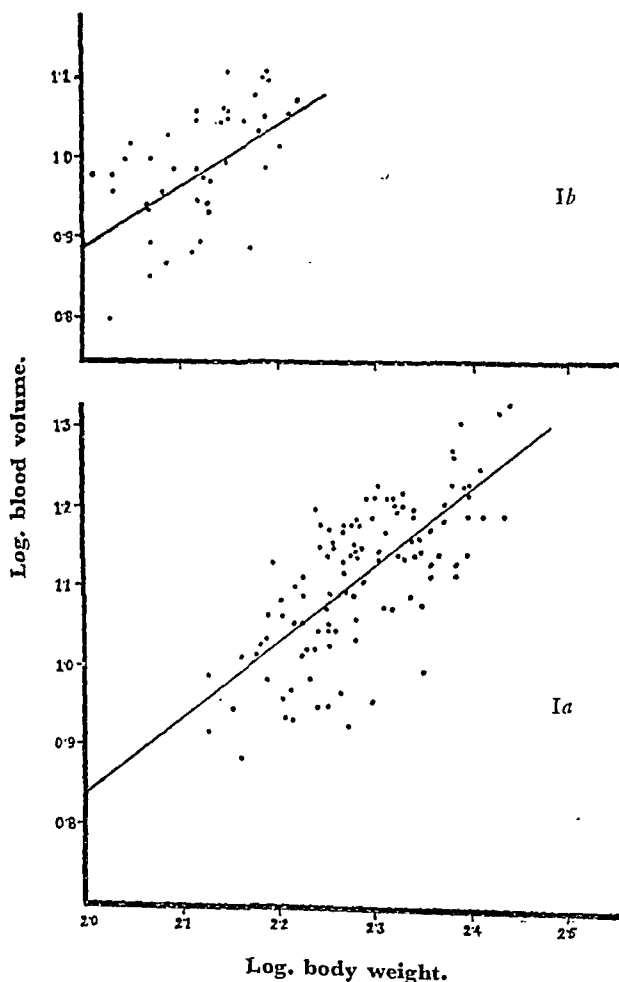
METHODS.

Blood volume was determined according to the method of Wang and Hegsted (1949) using Evans blue (T 1824) dye. The animal was anæsthetized by injecting intraperitoneally 50 mg./kg. body weight of sodium pentothal; 0.4 c.c. to 0.5 c.c. of the dye solution (5 mg./c.c. in normal saline) was then injected through the external jugular vein. Blood was drawn by cardiac puncture exactly after 4

minutes and the concentration of dye estimated according to the modification introduced by Clausen and Lifson (1956). Packed cell volume was determined by the method of Hamre (1940) using micro-hæmatocrit tubes. Hæmoglobin was determined by the acid hæmatin method after standardization of the technique. Total plasma proteins were estimated by the biuret method of Kingsley (1939) as modified by Weichselbaum (1946) using suitable blanks to eliminate the interference caused by the presence of Evans blue in the plasma.

A. *Blood volume in normal adult rats.*—Blood volume was determined in 107 normal young adult rats 80 to 130 days old and weighing between 120 g. and 260 g. The results are illustrated in Graph 1-a where the logarithms of blood

GRAPH 1.



Relation between blood volume and body weight in albino rats
 Ia—normal rats $y = 0.96x - 1.08$
 Ib—protein depleted rats $y = 0.73x - 0.57$

blood volume are plotted against those of body weight. The relationship between the two can be expressed by the regression equation $y = 0.96x - 1.08$ ($r = 0.72$) where x is the logarithm of body weight and y that of blood volume; this is indicated in the figure by the regression line. The relation between the two characteristics observed by us is not significantly different from that reported by Wang and Hegsted (*loc. cit.*) in rats ($t = 1.48$; D.F. 206).

B. *Protein depletion*.—Healthy rats, from stock colony, 41 in number, of the average age of 120 days and with average weight 200 g. were used for the experiment. They were fed *ad lib.* a protein-free diet otherwise complete in minerals and vitamins. The composition of the diet was as follows: Corn starch—90 parts, sesame oil—5 parts, salt mixture (McCollum and Davis, 1918)—4 parts, and vitamin mixture*—1 part. Vitamins A, D and E in sesame oil were fed to animals twice a week in adequate quantities†. Rats also received biotin 4 μ g. per day per rat twice weekly.

Blood volume, packed cell volume, hæmoglobin and plasma protein concentration were determined on all the animals before placing them on protein-free diets and on batches of animals at the end of 4, 8 and 10 weeks on protein-free diets. Since the determination of blood volume involved a minor operative procedure, each rat was used only twice for such determinations during the course of the experiment. The results of this experiment are given in Table I.

TABLE I.

Body weight, blood volume, plasma volume, hæmoglobin and plasma protein changes on a protein-free diet.

	INITIAL OBSERVATIONS:		ON PROTEIN-FREE DIET:					
	(41).		4 weeks (17).		8 weeks (15).		10 weeks (9).	
	Average.	S.E.	Average.	S.E.	Average.	S.E.	Average.	S.E.
Body weight g. ...	201	4.29	170	6.95*	131	4.47*	129	5.49*
Blood volume, c.c. ...	14.12	0.46	14.43	0.57	9.26	0.43*	9.20	0.62*
Plasma volume, c.c. ...	8.75	0.33	9.84	0.35	6.84	0.34*	6.78	0.44*
P.C.V. per cent ...	38.3	0.75	31.4	1.43*	26.2	1.16*	26.0	1.91*
Hæmoglobin, g./100cc.	13.69	0.14	12.61	0.24	10.00	0.33*	10.37	0.63*
Plasma protein, g./100 c.c. plasma ...	4.80	0.11	3.66	0.13*	3.40	0.09*	3.28	0.16*
Total circulating hæmoglobin, g. ...	1.946	0.08	1.822	0.08	0.931	0.07*	0.964	0.10*
Total circulating plasma protein, g. ...	0.416	0.02	0.358	0.02	0.232	0.01*	0.222	0.02*

* Indicates that deviations from initial values were significant ($P < 0.01$).

Figures in parenthesis indicate number of animals.

* *Composition of vitamin mixture*.—Thiamine hydrochloride 50 mg., riboflavin 100 mg., calcium pantothenate 100 mg., pyridoxine 25 mg., niacin 100 mg., choline chloride 5 g., folic acid 10 mg., para-aminobenzoic acid 1 g., inositol 1 g., vitamin B₁₂ 200 μ g., and sucrose 92.6 g.

† Dosage of vitamin A, 100 I.U., vitamin D 15 I.U., α -tocopherol acetate 1 mg. per week per rat.

It will be seen from Table I that body weights decreased progressively till the eighth week after which further fall till the tenth week was insignificantly small. The blood volume and plasma volume on the other hand changed little at the end of first four weeks, the plasma volume showed in fact a slight rise at this period. The concentration of hæmoglobin had decreased slightly in four weeks and although the plasma protein concentration showed a considerable decrease, there was little change in the amounts of total circulating plasma protein because of the increase in plasma volume. The real depletion in the total circulating hæmoglobin and plasma proteins became manifest at the end of 8 weeks at which period a marked decrease in absolute values of blood and plasma volumes also occurred. The average reduction in the circulating hæmoglobin and plasma proteins at 8 weeks was 56 per cent and 48 per cent respectively; hence this stage was considered suitable for commencing repletion with the test proteins.

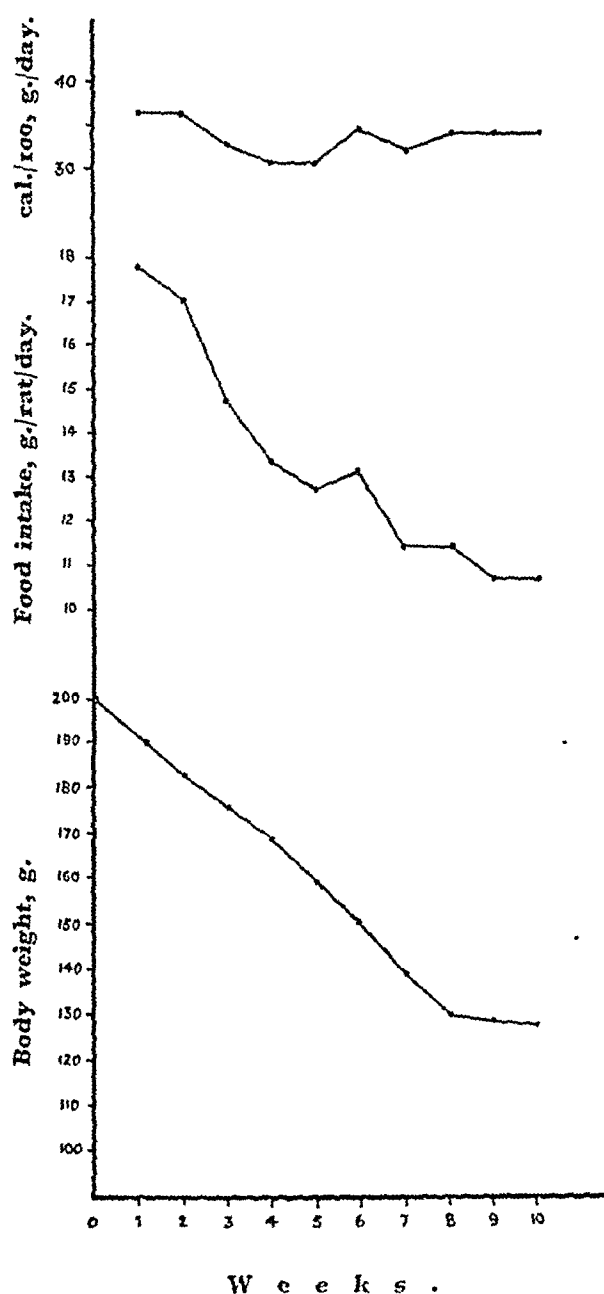
Reduction in the body weights of animals during the protein depletion period was accompanied by a corresponding fall in food intake as is illustrated in Graph 2. It was the lack of protein in the diet which was primarily responsible for the fall in body weights, food consumption keeping pace with this fall. The total average caloric intake of rats during depletion varied between 31.0 and 36.5 calories per 100 g. body weight and was sufficient to meet the total metabolic needs of the rats (Kibler and Brody, 1942). In view of this it is reasonable to assume that the blood changes described above were due to protein depletion alone and that inanition did not play any rôle in bringing them about.

Although the above experiment was primarily designed to standardize the conditions of depletion, the depleted animals were utilized for a preliminary repletion experiment. It was found that a majority of the depleted animals could not survive the operative procedure for the determination of blood volume and the mortality following the operation, minor though it was, was fairly high. Further, the healing of the surgically inflicted wound was considerably slow in protein depleted animals.

Since jugular veins were being utilized for injection of the dye it was considered undesirable for reasons stated above to use the same animal on more than two occasions during the course of the repletion experiment. This was only possible if the baselines for blood volume could be established not only for normal rats but also for animals depleted by feeding protein-free diets for a period of eight weeks. A second batch of 27 rats was, therefore, placed on protein-free diet for eight weeks and their blood volumes were determined. A regression equation indicating the relationship between body weight and blood volume was worked out for 42 rats (from two experiments) depleted of protein for 8 weeks. In fact, it was found that the regression equation expressing the relation between body weight and blood volume calculated for the normal adult rats was not significantly different ($t=1.15$; D.F. 145) from that for the protein-depleted rats (Graph 1-b) at eight weeks period. Advantage was taken of this observation to modify the procedure followed in the subsequent experiment as will be clear from the following description.

C. *Regeneration of hæmoglobin and plasma proteins.*—Forty eight male albino rats, 120 days old, with an average weight of 225 g. were taken from the stock colony and were fed protein-free diet as described above. At the end of 8 weeks,

GRAPH 2.

*Body weight and food intake of rats on protein free diets.*

hæmoglobin and plasma protein concentrations and packed cell volume were determined in each animal on blood samples taken by cardiac puncture. For reasons given above, values for blood volume in these animals were calculated from the regression equation for the protein-depleted animals. The rats were then divided into three groups and kept on the diets containing the following foodstuffs as sources of protein:

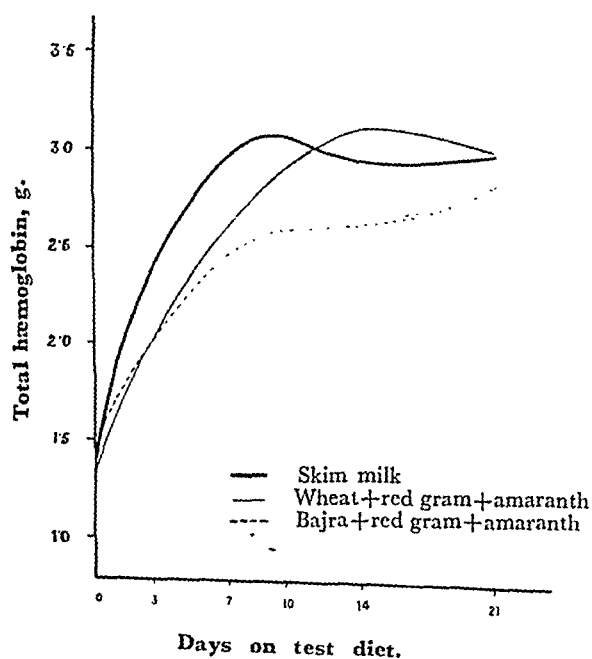
DIET 1— Source of protein—Skim milk (10 per cent protein).

DIET 2— „ „ Wheat (6 per cent protein)+red gram (3 per cent protein)+amaranth (1 per cent protein).

DIET 3— „ „ Bajra (6 per cent protein)+red gram (3 per cent protein)+amaranth (1 per cent protein).

The method of preparation of these diets and their composition have already been described by Phansalkar, Ramachandran and Patwardhan (*loc. cit.*). Animals in each group received 12 g. of the diet per day per rat which provided 49 calories and 1.2 g. protein per day. The intake of total protein in different groups and of calories was thus identical. The determinations of blood volume, packed cell volume, hæmoglobin and plasma protein concentrations were made in batches of rats from all the three groups 3, 7 and 10 days after the commencement of repletion. Two more observations were made at the end of 14 and 21 days utilizing the same rats which were used at 7 and 10 days period respectively. The results of this experiment are given in Table II. In Graphs 3 and 4 are given the smooth curves (fitted after statistical analysis), illustrating the regeneration of the total circulating hæmoglobin and plasma proteins, respectively, in the protein repletion period on all the three diets.

GRAPH 3.



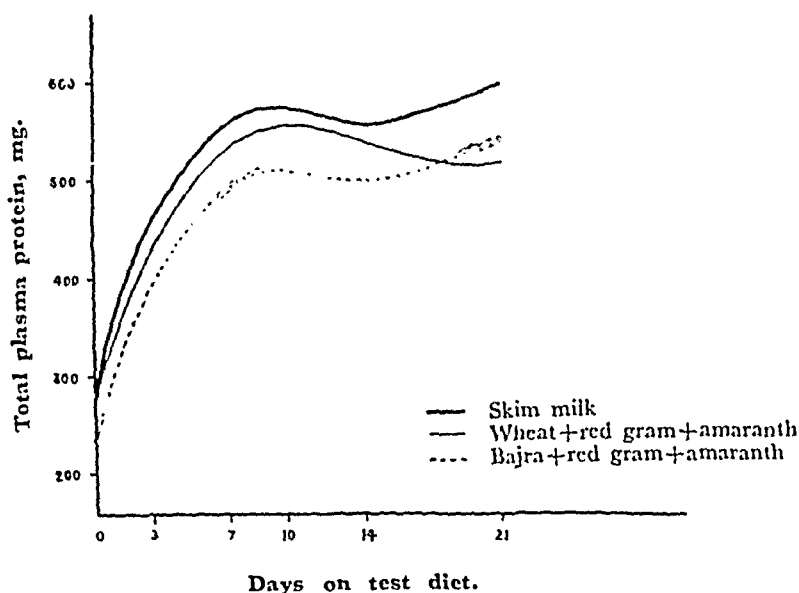
Regeneration of hæmoglobin during protein repletion.

TABLE II.

Changes in body weight, blood and plasma volumes, hemoglobin and total plasma protein during protein repletion.

Dietary protein.	SKIM MILK:							WHEAT + RED GRAM + AMARANTH:							BAJRA + RED GRAM + AMARANTH:						
	Days:							Days:							Days:						
	Initial.	3	7	10	14	21	Initial.	3	7	10	14	21	Initial.	3	7	10	14	21			
Body weight, g.	171	173	176	197	186	202	167	166	185	191	192	198	157	152	178	179	187	187			
Blood volume, c.c.	11.51	19.81	20.13	25.45	20.39	21.83	11.28	18.88	20.62	21.64	20.47	21.09	10.85	19.70	17.20	20.80	18.88	20.46			
Plasma volume c.c.	7.84	13.68	12.71	16.31	12.46	13.50	7.92	13.21	13.44	13.99	14.15	13.18	7.43	13.92	11.23	13.65	11.97	12.63			
P.C.V. per cent	31.8	31.0	37.0	35.7	39.0	38.2	29.7	30.2	34.4	35.1	37.0	40.0	31.5	29.5	34.2	34.1	36.8	38.2			
Hemoglobin, g./100 c.c.	12.82	11.81	14.26	13.83	13.94	14.87	12.69	11.26	12.81	13.59	13.25	15.46	12.84	10.06	13.94	13.33	13.40	14.52			
Plasma proteins, g./100 c.c.	3.33	3.45	4.06	3.74	4.38	4.43	3.23	3.58	3.73	3.88	3.96	3.93	3.08	3.40	3.76	3.69	4.08	4.25			
Total circulating hemoglobin, g.	1.453	2.358	2.862	3.301	2.843	2.995	1.427	2.131	2.665	2.951	2.972	3.056	1.393	1.965	2.403	2.779	2.523	2.837			
Total circulating plasma protein, g.	0.263	0.470	0.518	0.610	0.542	0.596	0.255	0.473	0.502	0.537	0.558	0.515	0.227	0.438	0.461	0.502	0.513	0.537			
Number of animals.	14	4	4	6	4	4	16	4	5	7	5	6	18	4	5	9	5	8			

GRAPH 4.



Regeneration of total plasma protein during protein repletion.

DISCUSSION.

Blood volume and plasma volume changes.—It will have been noticed that during protein depletion blood volume altered little at first but showed a precipitous fall between 4 and 8 weeks (Table I). This trend was reversed in the same order during repletion. Blood volume increased by 67 to 82 per cent and plasma volume by 67 to 87 per cent over the depletion levels within 3 days of the start of protein repletion. Increases in both these values in the subsequent period up to 21 days were comparatively small. On the other hand, body weights of rats increased steadily on all the three diets throughout the experimental period, with the result that at 3 days, blood and plasma volumes in relation to body weights were higher than could be expected from the recorded body weights. This disparity tended to decrease but was not altogether eliminated even after 20 days of repletion when the total circulating haemoglobin and plasma proteins had returned to normal. Mollison (1946), Walters, Rossiter and Lehman (1947) and Denz (1947) have observed similar changes in malnourished human subjects during the early stages of protein repletion.

Changes in haemoglobin.—It will be seen from Table II that haemoglobin concentration at the end of 21 days of repletion increased between 13·8 and 21·8 per cent over the depletion levels. Three days after the start of repletion haemoglobin concentration actually showed a fall which is attributable to a sudden rise in the blood volume. Total circulating haemoglobin, however, showed an increase in all the groups even at this period. It must be said, therefore, that at any stage a truer picture of regeneration is given by the total circulating haemoglobin.

Increases in its levels at 21 days period were of the order of 100 to 109 per cent over the depletion levels. It is clear, therefore, that on all the three diets rats could double the quantity of the circulating hæmoglobin indicating that the two vegetable protein mixtures were equally efficient for hæmoglobin regeneration. The rate of regeneration, however, was quickest with skim milk, the peak being reached within ten days, that on wheat diet was reached in 14 days and in 21 days on bajra diet.

Changes in plasma protein.—The average plasma protein concentration increased in 21 days on different dietary proteins by 22 to 38 per cent above the depletion levels. Expressed in the terms of the total circulating plasma protein, the increases were 97 to 122 per cent. The rates of plasma protein regeneration were different on the three experimental diets, that on skim milk being the most rapid and on bajra diet the slowest, as in the case of hæmoglobin. This might be a possible explanation of the lower values of serum albumin observed by Venkatachalam *et al.* (1956) in kwashiorkor cases treated with vegetable proteins on the tenth day of treatment as compared with that obtained on treatment with skim milk during the same period. Since in the present experiment, the vegetable protein mixtures used in this investigation were found capable of restoring the plasma protein levels of the depleted animals to normal, it would be reasonable to assume that longer periods of treatment would be necessary to obtain a similar effect in cases of kwashiorkor which are treated with vegetable proteins. The sudden changes in the plasma volume observed in the early stages of repletion influence, as in the case of hæmoglobin, the concentration of plasma proteins by masking the extent of the regeneration of the latter. Allison (1948) had observed similar changes in protein-depleted dogs during the repletion period. In his series, the average total circulating plasma proteins in depleted dogs were 6.4 g. which rose on repletion to 15.6 g. although the plasma protein concentrations at the corresponding periods were 5.9 and 6.1 g. per 100 c.c. plasma.

General.—It has been stated before that body weights increased steadily. There were no differences in the rates of gain on the three diets. However, the body weights did not reach the initial level which obtained before depletion. Only about 50 per cent of the weight lost was regained during the process of repletion on all diets. Restriction of food intake to 12 g. per day was probably responsible for this. The total circulating hæmoglobin and total circulating plasma proteins, however, reached the predepletion levels apparently taking priority over body weight gain. This appears to be just the reverse process of what was observed in the depletion period, viz. the fall in body weight occurred earlier than the fall in the total circulating hæmoglobin and plasma proteins.

Another fact which deserves mention is that at all stages of regeneration and on all the three experimental diets, the regenerated hæmoglobin formed 76 to 86 per cent of the newly formed total circulating blood proteins, the rest being plasma proteins. Whipple and Robscheit-Robbins (1951) have observed that dietary proteins differed in their capacity to regenerate hæmoglobin and plasma proteins. The regeneration of the two protein moieties in their doubly depleted dogs was in the proportion of 2 g. hæmoglobin to 1 g. plasma protein when fed casein diets. More plasma protein than hæmoglobin was produced on diets containing egg proteins, egg albumin and lactalbumin. No such differences were observed in the

present series of experiments. Whipple and Robschey-Robbins (1951) further report that wheat gluten, soya bean flour and peanut—when used as single sources of protein proved inadequate for the regeneration of blood proteins. It is difficult to compare their results with those described in the present communication on account of differences in experimental procedures. It is possible that the above mentioned vegetable proteins used singly were inefficient for the regeneration of blood proteins, or, as our results would suggest, the response could have been at a much slower rate than that obtained with casein.

Conclusion.—It can be concluded from the results discussed in the preceding pages that although the vegetable protein mixtures used in this investigation promoted regeneration of haemoglobin and plasma proteins at slightly slower rates than skim milk they did not lack the capacity to regenerate them fully if given sufficient time. In view of our earlier observation (Phansalkar, Ramachandran and Patwardhan, *loc. cit.*) that these protein mixtures maintained normal haemoglobin and plasma protein concentrations in growing rats, and their capacity (now clearly demonstrated) to regenerate blood proteins which is not much lower than that of skim milk, it would be safe to assume that cereal-pulse-leafy vegetable diets have a place in the treatment as well as the prevention of protein malnutrition.

SUMMARY.

1. The effect of depleting body stores of proteins on blood and plasma volumes, haemoglobin and plasma proteins was studied in adult albino rats on protein-free diets.

2. The capacity of two vegetable protein mixtures—(a) bajra, red gram and amaranth, and (b) wheat, red gram and amaranth to regenerate blood proteins was determined in protein-depleted albino rats over a period of 3 weeks using skim milk protein as control.

3. On protein-free diets, rats lost body weight continuously till the eighth week after which the fall in body weight was inconsiderable. On the other hand, blood and plasma volumes did not appreciably alter in the first four weeks of depletion, but were considerably reduced in the subsequent four-week period. The reduction in the total circulating haemoglobin and plasma protein was 56 per cent and 48 per cent respectively after eight weeks on the protein-free diet.

4. During repletion at 10 per cent protein intake level, blood proteins tended to return to normal on all the three diets. The rate of regeneration in animals on the vegetable protein diets, however, was lower than on skim milk diet.

5. The rate of gain in body weight was almost the same on the three diets. The animals did not regain their initial weight within three weeks of repletion, although the values of total circulating haemoglobin and plasma proteins were restored to normal. These results suggest that the regeneration of blood proteins took priority over the restoration of body weight.

6. The results indicate that suitable vegetable protein combinations could be used for the prevention and treatment of protein malnutrition and the anaemia associated with it.

The investigation forms a part of the Protein Malnutrition Project, funds for which were provided by the World Health Organization. The authors have great pleasure in expressing their gratitude for this assistance which enabled two of them (S.V.P. and M.R.) to participate in the investigation. The authors are also grateful to Dr. A. D. Taskar for his expert assistance in the statistical aspects of the investigation.

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AMINO-ACID COMPOSITION OF EDIBLE MUSCLES.

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THE qualitative and quantitative occurrence of sixteen common amino acids in the muscle tissues of a variety of warm and cold blooded indigenous animals have been studied by means of paper-chromatographic and microbiological-assay methods. The investigation has been pursued with the idea that the data of this type might demonstrate the relative values of different meats in supplying the nutritionally significant constituents. Incidentally, paper-chromatographic methods, as used for the estimation of amino acids, have been appraised by comparing the observed distribution with these obtained by microbiological assay methods. In the choice of animals, special attention has been paid to the meats eaten by the hunter tribes of Maharashtra. No detailed report appears to be available regarding the meats chosen for the study. The following animals have been chosen as representative of the respective classes to which they belong:—

Class.	Scientific name.	Local name.
Mammal	<i>Hystrix leucura</i>	Salu
Mammal	<i>Lepus nigricolis</i>	Sesa
Mammal	<i>Bandicota indicus</i>	Ghoose
Aves	<i>Francolinus pondicerianus</i>	Chittar
Aves	<i>Bubulcus ibis</i>	Bagala
Aves	<i>Fulica atra</i>	Pan-kombadi
Reptile	<i>Varanus monitor</i>	Ghorpad
Reptile	<i>Lissemys punctata</i>	Kasav
Amphibia	<i>Rana tigrina</i>	Beduk
Fish	<i>Macrones cavasius</i>	Shingti

The analyses of amino acids from different sources have shown that meats, in general, contain all of the indispensable amino acids in liberal amounts and that the proteins of the meat are biologically complete. Beach *et al.* (1943) have observed that protein structure which makes up of voluntary muscle tissues is similar in animals.

With the advent of microbiological assay methods, increased efforts have been devoted to the determination of amino-acid composition of meats. Schweigert, Tamtan and Elvehjem, 1945; Greenhut, Schweigert and Elvehjem, 1946; Greenhut, Sirney and Elvehjem, 1948; and Greenhut, Schweigert and Elvehjem, 1946, have determined a number of amino acids from lamb, pork, beef, and veal. Leucine, isoleucine and valine contents of these averaged 7.7, 5.7 and 5.2 per cent, respectively. The values obtained for tryptophan and phenyl alanine were 1.3 and 3.9 per cent, respectively. The contents of methionine, arginine and histidine ranged between 2.2 and 3.3 per cent, 6.4 and 6.5 per cent and 3.1 and 3.4 per cent while threonine and lysine averaged 4.3 per cent and 8 per cent, respectively.

The contents of ten indispensable amino acids in fresh and cooked pork and lamb have been reported (Schweigert *et al.*, 1949). A thorough microbiological assay of 55 meat samples (beef, lamb, pork, veal and various beef organs) for arginine and histidine gave further proof to the concept that specific amino-acid content of proteins of comparable animal tissues is similar (Sirney, Greenhut and Elvehjem, 1950). The amino-acid content of proteins of several commercial fishes and their by-products have also been estimated by microbiological-assay methods (Ney *et al.*, 1950; Dunn *et al.*, 1949; Seagran *et al.*, 1954; and Master and Magar, 1954).

In the present research, most of the amino acids resulting from protein hydrolysis have been identified and estimated on paper chromatograms following the modifications of circular paper chromatography as proposed by Giri and Rao (1952) and Krishnamurthy and Swaminathan (1955). Paper-chromatographic methods have been employed to estimate, arginine, alanine, tyrosine, phenylalanine, lysine, glutamic acid, threonine, aspartic acid, serine, and glycine, whereas microbiological assay methods have been used to determine tryptophan, leucine, isoleucine, valine, methionine, lysine, histidine, phenyl alanine, arginine and threonine.

EXPERIMENTAL METHODS AND COMPUTATION OF RESULTS.

Hydrolysis.—For paper chromatographic work, requisite amounts of dried and defatted muscles (containing about 32 mg. of nitrogen) were hydrolysed, with 20 c.c. of 6N HCl under reflux for 18 to 24 hours according to the method of Block (1955). At the end of the hydrolysis period, the contents of the hydrolysing flask were transferred to an evaporating dish and excess of HCl was removed. The hydrolysate was then dissolved in 25 c.c. of water and filtered. The filtrate was evaporated to dryness on a steam-bath and the residue was dissolved in 10 c.c. of 10 per cent aqueous isopropanol. The hydrolysate was further diluted before use.

For microbiological assay of amino acids, hydrolytic procedures with 2, 5N HCl were used as recommended by Barton-Wright (1952) except for tryptophan for which alkaline hydrolysis with barium hydroxide was used.

Estimation of nitrogen.—Total nitrogen in the dried and defatted samples was determined by micro-Kjeldahl method.

Paper chromatography.—Estimations of arginine, alanine, tyrosine and phenyl alanine were carried out on circular paper chromatograms by elution of the separated bands and subsequent colorimetry (Giri *et al.*, 1952). The estimations of lysine, glutamic acid, threonine, aspartic acid, glycine and serine were carried out following the buffer technique as modified by Krishnamurthy and Swaminathan (*loc. cit.*). Each amino acid was determined in triplicates on two different chromatograms.

Microbiological assays.—Microbiological assay methods as recommended by Barton-Wright (*loc. cit.*) were carried out to assay tryptophan, leucine, isoleucine, valine, lysine, methionine, phenyl-alanine, histidine, arginine and threonine. *Lactobacillus arabinosus* 17 to 5 was used for the assay of tryptophan, leucine, isoleucine, and valine. Lysine, phenyl alanine and histidine and methionine were estimated using *Leuconostoc mesenteroides* p. 60, while threonine and arginine were assayed using *Streptococcus faecalis* (letis R.).

RESULTS.

In Table II are given the percentage of amino acids. Cystine and histidine could not be estimated on paper-chromatograms as the colorimetric readings for these amino acids were rather low. In the case of tyrosine, the band corresponding to this amino acid occurred just above the yellow band of proline, in butanol acetic acid-water system with the result that there was a considerable interference of proline with tyrosine estimations.

All the microbiological assay results were calculated by Wood's statistical method. When the logarithm of the dose levels was plotted against their response, a straight line was obtained both for standards as well as for test preparations. For the assay of tryptophan, complete racemization was assumed and hence the values were doubled to correct for racemization.

In Table I are given the moisture, nitrogen and ash contents of the edible muscles.

TABLE I.
Showing the percentages of moisture nitrogen and ash contents of edible muscles.

Animal.	Nitrogen*.	Moisture.	Ash.
<i>Hystrix</i> ...	14.60	75.27	0.90
<i>Lepus</i> ...	14.20	72.34	1.11
<i>Bandicota</i> ...	13.68	74.00	1.30
<i>Francolinus</i> ...	14.60	72.65	1.16
<i>Bubulcus</i> ...	14.20	72.10	1.21
<i>Fulica</i> ...	14.07	75.60	1.01
<i>Varanus</i> ...	14.07	72.47	1.18
<i>Lissemys</i> ...	13.80	71.84	0.98
<i>Rana</i> ...	14.73	75.20	0.87
<i>Macrones</i> ...	14.07	80.34	0.87

* In dried and defatted muscles.

TABLE II.

Amino-acid composition of edible muscles calculated to 16 per cent nitrogen.

Amino acid.	<i>Hystrix.</i>	<i>Lepus.</i>	<i>Baudicola.</i>	<i>Francolinus</i>	<i>Bubulcus.</i>	<i>Fulica.</i>	<i>Varanus.</i>	<i>Lissonys.</i>	<i>Rana.</i>	<i>Macroneis.</i>
Arginine* ...	5.90	6.05	5.75	6.00	5.90	5.90	5.95	6.10	5.85	6.25
Arginine† ...	4.82	5.19	4.91	4.82	5.31	5.36	4.89	4.99	5.24	5.59
Alanine* ...	7.25	7.45	6.50	6.85	6.05	6.00	7.15	7.35	6.75	6.85
Tyrosine* ...	6.05	6.10	5.35	5.70	5.80	6.15	5.25	5.75	6.00	6.00
Phenyl alanine*	5.45	5.45	5.35	5.15	5.45	5.20	5.25	5.45	5.15	5.40
Phenyl alanine†	3.28	3.45	3.34	3.21	3.78	3.82	3.40	3.55	3.91	3.82
Lysine* ...	8.25	8.75	9.10	8.65	8.55	8.75	9.10	8.70	8.90	9.40
Lysine† ...	9.77	10.28	9.73	9.99	9.48	9.57	10.52	10.33	9.91	10.37
Methionine†	3.07	2.94	2.80	3.20	3.32	3.18	2.71	2.88	3.32	3.31
Glutamic acid*	13.60	13.95	14.45	14.40	14.90	14.65	13.95	15.30	13.65	14.50
Threonine*	5.95	6.05	6.10	5.50	5.95	5.85	5.80	5.80	5.55	6.20
Threonine†	3.94	4.05	4.02	3.94	4.25	4.19	4.04	4.08	4.05	4.29
Aspartic acid*	7.70	7.40	8.65	7.70	8.20	7.25	8.35	8.35	8.85	8.75
Serine* ...	4.60	4.30	4.95	4.15	4.95	5.05	4.30	4.65	4.70	5.00
Glycine* ...	5.60	5.35	6.00	5.90	6.45	5.45	6.45	6.45	5.80	6.15
Tryptophan†	1.04	1.04	1.04	0.96	1.04	1.01	1.01	1.02	1.00	1.08
Lecucine† ...	7.43	7.98	7.39	7.44	7.64	7.55	7.45	7.68	7.76	7.96
Isoleucine†	5.85	4.99	5.12	4.85	4.82	4.81	5.33	5.19	5.10	5.20
Valine† ...	4.91	5.16	5.01	4.80	4.94	4.98	4.98	4.82	4.94	5.68
Histidine† ...	2.26	2.41	2.24	2.19	2.45	2.42	2.39	2.68	2.67	2.38

* Paper-chromatographic.

† Microbiological assay.

DISCUSSION.

In Table II are given the percentages of amino acids calculated to 16 per cent nitrogen, as obtained by paper-chromatographic and microbiological-assay methods. Comparison between the above methods both of which were employed for the estimation of lysine, phenyl alanine, arginine and threonine, indicate that the former method gave in general slightly higher values than the latter. While the microbiological-assay methods are supposed to give accuracy equal to that of any other analytical method (Giri *et al.*, *loc. cit.*), the paper-chromatographic methods also compare favourably with microbiological methods, thus indicating that paper-chromatographic methods may also prove equally useful for the routine estimation of amino acids.

In general, the values for different amino acids obtained by paper-chromatographic methods also compare well with the values reported in literature for muscle tissues. In the case of tyrosine, however, the values obtained by this method were slightly higher than the corresponding values in literature, even though acid hydrolysates which are not generally recommended for tyrosine, were used. This is because of the fact that there was a considerable interference of proline with tyrosine estimations, since these two amino acids occurred very near each other on chromatograms prepared with butyl alcohol-acetic acid-water.

Comparison of values obtained by microbiological-assay methods with those of beef, veal, pork and lamb as obtained by Schweigert, Tamtan and Elvehjem, *loc. cit.*; Greenhut, Schweigert and Elvehjem, *loc. cit.*; and Greenhut, Sirney and Elvehjem, *loc. cit.*, shows that while the values of tryptophan, leucine, isoleucine, valine, phenyl alanine and threonine compare favourably, the values for histidine and arginine obtained in the present work were lower whereas lysine values were slightly higher.

The difference in histidine values may be explained on the basis of carnosine content. Lyman, Kuiken and Hale (1947) and Lyman Kuiken (1949) have made a thorough investigation of similar differences which they observed in meats and have attributed them to the occurrence of histidine in the muscles in the non-protein form of carnosine. Thus, it may mean that the animals under investigation may contain less carnosine in the muscle tissues, than pork, lamb or veal. In the same way *Lissemys* and *Rana* with a histidine content of about 2.7 per cent may have more carnosine in their muscle tissues than in the muscles of other animals investigated.

A rather wide disagreement exists among the values for arginine in literature ranging from 3.4 to 7.9 per cent (Greenhut, Schweigert and Elvehjem, *loc. cit.*). However, arginine content of about 5 per cent as obtained in the present work is in good agreement with that of Hart (1901) who reported a content of 5.1 per cent for arginine in ox muscle. Similarly, the values for other amino acids are also in conformity with the values reported by Block and Bolling (1951) for animal muscles in general.

Thus, from the standpoint of protein quality, as determined by amino-acid content, the edible muscles from indigenous animals under investigation must be considered to contain protein of the finest nutritive quality, equalling to that of beef, veal, pork, chicken and other common meats.

It is also evident from Table II that the general pattern of amino-acid distribution of the sixteen amino acids in muscles from mammals, aves, reptiles, amphibia and fish appear to be similar. Similarity of amino-acid composition of muscles from different animal species have also been reported (Beach *et al.*, *loc. cit.*, Greenhut, Schweigert and Elvehjem *loc. cit.*, and Block and Bolling, *loc. cit.*). Findings such as these might suggest that the structure or amino-acid make-up of proteins of voluntary muscles in different vertebrate animals is essentially the same. Such a concept is in agreement with Beach *et al.* (*loc. cit.*) who found marked similarity of essential amino-acid composition of muscles of some animal species.

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A STUDY OF THE TECHNIQUE FOR DEMONSTRATION OF 'O' AND 'H' AGGLUTININS IN THE SERUM OF CHOLERA PATIENTS.

BY

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INTRODUCTION.

BACTERIOLOGICAL diagnosis of cholera is not always satisfactory. Cholera is a self-limited disease provided the crisis of shock is managed properly, and the vibrios in the stool tend to diminish and disappear more or less quickly after the acute episode. Moreover, there may be milder cases where the specific vibrios may appear in stool in such small numbers as cannot be isolated by culture. This led us to think if serological study may be of some use in retrospective diagnosis of cholera, particularly from an epidemiological point of view. Search of the relevant literature failed to give clear-cut information on the detection of O and H agglutinins of *Vibrio cholerae* in the sera of patients. Therefore, we had to concentrate at the outset on finding out such a method.

MATERIALS AND METHODS.

Fifty samples of blood sera were obtained from fifty culture positive cases of cholera between the fifth and the seventh day of the disease. The presence or absence of agglutinins against (1) Ogawa type of *Vibrio cholerae*, (2) *Bacterium coli*, (3) *B. lactis aerogenes*, (4) *B. enteritidis*, (5) *Brucella melitensis*, (6) *Brucella abortus*, and (7) *B. faecalis alkaligenes*, in the sera was checked by slide method using saline

suspensions of twenty-four hour agar culture of those organisms as antigens. This was followed by Dreyer's-tube method in which four separate antigens were used, viz., saline suspension of live cholera vibrio (Ogawa type) grown for twenty-four hours on nutrient agar slope; saline suspension of agar culture of a non-agglutinating vibrio having the same H antigen (but different O antigen) as *Vibrio cholerae*; formalised (0.25 per cent) broth culture of *Vibrio cholerae* (Ogawa); and formalized (0.25 per cent) broth culture of the same non-agglutinating vibrio as above. All the antigens were filtered through Whatman filter paper (No. 1) to remove the coarser clumps and were diluted to the density of about 1000×10^6 organisms per c.c. The saline suspensions were prepared fresh each day and the formalized broth cultures were obtained from stock preserved at 4°C. Three dilutions, viz., 1 in 25, 1 in 83 and 1 in 250 of the sera were used with each antigen. One set was incubated at 37°C. in an incubator and another set was immersed in a water bath at 56°C. Saline controls were put up for each antigen with each set. Readings were taken against shaded bright light after $\frac{1}{2}$, 1, 2 and 4 hours and also on the next morning (about 22 hours).

RESULTS.

All the fifty sera gave positive slide agglutination with *Vibrio cholerae* (Ogawa) and none with any of the other six organisms tested. Regarding tube agglutinations with the four antigens used, the fifty specimens showed one of five types of reactions: Type I reaction was given by fourteen specimens, type II by six specimens, type III by eighteen, type IV by ten and type V by two specimens of sera. A representative reaction of each type is given below:—

Type I reaction.—When incubated at 37°C. agglutination appeared with living suspension of *V. cholerae* in two hours in all the tubes except in the first one (zone-phenomenon), which, however, showed appearance of clumping in four hours. No agglutination was seen with formalized *V. cholerae* even after twenty-two hours. With NAG formalized suspension faint agglutination appeared in the first tube in four hours but in all the tubes in twenty-two hours.

When incubated at 56°C., evidence of clumping was seen with living suspension of *V. cholerae* not before four hours when all the tubes were positive. With formalized *V. cholerae*, agglutination appeared in the first two tubes (definite in first tube and faint in the second tube) in two hours and in all the tubes by four hours. With formalized NAG suspension fine clumps were seen in two hours in the first tube, while this was more definite in four hours, but all the tubes showed evidence of clumping in twenty-two hours. These results are set out in Table I.

Type II reaction.—When incubated at 37°C. clumping appeared with living suspension of *V. cholerae* in all the tubes in two hours and with formalized *V. cholerae* in the first tube in two hours and rather faintly in the second tube after four hours. Agglutination was, however, seen in all the tubes after twenty-two hours. With formalized NAG suspension hours, agglutination was apparent after four, in the first tube and more faintly in the second tube, in which the reaction became definite after twenty-two hours.

When incubated at 56°C. no agglutination appeared with living suspension of *V. cholerae* in any of the tubes even after four hours. With formalized *V. cholerae*

agglutination appeared in all the three tubes after two hours. With formolized NAG suspension, faint agglutination appeared in the first tube after two hours, which became definite after four hours and in the second tube after twenty-two hours (Table II).

TABLE I.

Showing type I agglutination reaction of cholera sera with different antigens at 37°C. and 56°C.

ANTIGEN.		<i>V. cholera</i> saline suspension.			<i>V. cholera</i> formolized.			NAG formolized.		
Temperature.	Hours.	I 25	I 83	I 250	I 25	I 83	I 250	I 25	I 83	I 250
37°C.	2	—	+	+	—	—	—	—	—	—
	4	+	+	+	—	—	—	f	—	—
	22	+	+	+	—	—	—	+	+	+
56°C.	2	—	—	—	+	f	—	f	—	—
	4	+	+	+	+	+	+	+	—	—
	22	+	+	+	+	+	+	+	+	+

TABLE II.

Showing type II agglutination reaction of cholera sera with different antigens at 37°C. and 57°C.

ANTIGEN.		<i>V. cholera</i> saline suspension.			<i>V. cholera</i> formolized.			NAG formolized.		
Temperature.	Hours.	I 25	I 83	I 250	I 25	I 83	I 250	I 25	I 83	I 250
37°C.	2	+	+	+	+	—	—	—	—	—
	4	+	+	+	+	f	—	+	f	—
	22	+	+	+	+	+	—	+	+	—
56°C.	2	—	—	—	+	+	+	f	—	—
	4	—	—	—	+	+	+	+	—	—
	22	—	—	—	+	+	+	+	+	—

Type III reaction.—When incubated at 37°C. with living suspension of *V. cholera*, agglutination appeared in the first and second tubes in two hours but none in the third tube even after four hours. Formolized *V. cholera* showed faint agglutination in the first tube after two hours, which was definite after four hours up to the second tube, but no agglutination was seen in the third tube even after twenty-two hours. With NAG formolized suspension, no agglutination appeared in any of the tubes even after twenty-two hours.

354 *Technique for Demonstration of Agglutinins of Cholera Patients.*

When incubated at 56°C. with living suspension of *V. cholera*, agglutination appeared in the first and second tubes in two hours but none in the third tube even after four hours. With formolized *V. cholera*, there was agglutination in the first and in the second tubes in two hours but none in the third tube even after twenty-two hours. With NAG formolized suspension, clumps were seen in the first tube in two hours but none in the second and third tubes even after twenty-two hours (Table III):—

TABLE III.

Showing type III agglutination reaction of cholera sera with different antigens at 37°C. and 56°C.

ANTIGEN.		<i>V. cholera</i> saline suspension.			<i>V. cholera</i> formolized.			NAG formolized.		
Temperature.	Hours.	I 25	I 83	I 250	I 25	I 83	I 250	I 25	I 83	I 250
37°C.	2	+	+	—	f	—	—	—	—	—
	4	+	+	—	+	+	—	—	—	—
	22				+	+	—	—	—	—
56°C.	2	+	+	—	+	+	—	—	—	—
	4	+	+	—	+	+	—	+	—	—
	22				+	+	—	+	—	—

Type IV reaction.—When incubated at 37°C. with suspension of living *V. cholera*, agglutination appeared in the first tube only in four hours. No agglutination appeared even after twenty-two hours either with formolized *V. cholera* or with formolized NAG culture.

When incubated at 56°C. with living suspension of *V. cholera*, no agglutination appeared in any of the tubes even after four hours. With formolized *V. cholera*, agglutination appeared in the first two tubes in two hours and in the third tube after twenty-two hours. With NAG formolized suspension, agglutination appeared in the first tube and fine clumping in the second tube after four hours but after twenty-two hours definite agglutination appeared in all the three tubes (Table IV).

Type V reaction.—When incubated at 37°C. with living suspension of *V. cholera*, faint agglutination appeared in the first tube after two hours and definite agglutination in the first and second tubes after four hours. With formolized *V. cholera*, faint agglutination appeared in the first tube in four hours which became definite after twenty-two hours but none in the second and third tube. With formolized NAG culture, no agglutination appeared in any of the tubes even after twenty-two hours.

When incubated at 56°C. no agglutination appeared in any of the tubes with *V. cholera* (living suspension) even after four hours. With formolized *V. cholera* agglutination was noted in the first two tubes in two hours but none in the third

tube even after twenty-two hours. With formalized NAG culture no agglutination appeared in any of the tubes even after twenty-two hours (Table V):—

TABLE IV.

Showing type IV agglutination reaction of cholera sera with different antigens at 37°C. and 56°C.

ANTIGEN.		<i>V. cholera</i> saline suspension.			<i>V. cholera</i> formalized.			NAG formalized.		
Temperature.	Hours.	I 25	I 83	I 250	I 25	I 83	I 250	I 25	I 83	I 250
37°C.	2	—	—	—	—	—	—	—	—	—
	4	+	—	—	—	—	—	—	—	—
	22	—	—	—	—	—	—	—	—	—
56°C.	2	—	—	—	+	+	—	—	—	—
	4	—	—	—	+	+	—	+	f	—
	22	—	—	—	+	+	+	+	+	+

TABLE V.

Showing type V agglutination reaction of cholera sera with different antigens at 37°C. and 56°C.

ANTIGEN.		<i>V. cholera</i> saline suspension.			<i>V. cholera</i> formalized.			NAG formalized.		
Temperature.	Hours.	I 25	I 83	I 250	I 25	I 83	I 250	I 25	I 83	I 250
37°C.	2	f	—	—	—	—	—	—	—	—
	4	+	+	—	f	—	—	—	—	—
	22	—	—	—	+	—	—	—	—	—
56°C.	2	—	—	—	+	+	—	—	—	—
	4	—	—	—	+	+	—	—	—	—
	22	—	—	—	+	+	—	—	—	—

All the specimens of group 3 showed evidence of agglutination to titre with living suspension of *V. cholera*, at both temperatures within half an hour, while the others did not. With living NAG suspension, no agglutination was observed in any tube at either temperature with any serum even after twenty-two hours.

DISCUSSIONS.

The results of the experiments may be analysed to arrive at a suitable method of detecting and titrating the O and H agglutinins of *V. cholera* in the serum of a person. In deciding this, it is worthwhile to remember that the patient's serum

may contain both O and H agglutinins of *V. cholerae*; the antigens prepared from *V. cholerae* carry both O and H agglutinogens of *V. cholerae* and those from the non-agglutinating (NAG) vibrios used by us carry only the H agglutinin (and not O) of *V. cholerae*. None of the fifty samples of sera agglutinated the NAG suspension either at 37°C. or at 56°C. This suggests that when using living saline suspension as the antigen in the agglutination, H agglutinin fails to combine with its corresponding antibody, which as we shall see later, was present in most of the sera, except those showing the type V reaction. Therefore, positive tube agglutination with living suspension of *V. cholerae* will indicate the presence only of O agglutination. Besides the clumps found in these cases were fine and granular and quickly sank to the bottom which is also suggestive of O agglutination. It may also be observed that for detecting O agglutination of *V. cholerae* with saline suspension of the organism, incubation at 37°C. is more dependable than at 56°C. Although agglutination to titre was seen within half-an-hour at both these temperatures in the reaction of type III, it did not appear even in four hours at 56°C. in those of types II, IV and V. In type I reaction such O agglutination did not appear at 56°C. before four hours, although at 37°C. it was seen in two hours. The occurrence of zone phenomenon in the reaction of type I deserves notice. Regarding the minimum times after which reading should be taken for O agglutination, the maximum positive titre could be obtained within half hour in reaction of type III, but there were others (type IV) where no agglutination was observed before four hours. Overnight readings with living suspensions is rendered difficult by development of turbidity due to growth of secondary organisms. Therefore, O agglutination should be read after four hours' incubation at 37°C. with saline suspension of living cholera vibrios.

H-agglutination.—Non-agglutinating vibrio with same H antigen as *V. cholerae* in the form of formalized broth culture, was agglutinated by all the sera of the cholera patients at 56°C., except by those showing type V reaction.

That this agglutination was not caused by O antigen of the non-agglutinating vibrio is indicated by absence of any agglutination with the live suspensions of the organisms either at 37°C. or at 56°C. Moreover, the clumps were more loose and took longer time to settle. At 56°C., H agglutination was positive in all types of cases except in type V, while at 37°C. it was positive only in types I and II. Therefore, incubation at 56°C. appears to be more suitable for H agglutination.

H agglutination appeared in some cases as early as two hours but a higher titre could be read after twenty-two hours than after four hours in types I, II and, IV. Thus, overnight incubation at 56°C. with formalized broth culture of a non-agglutinating vibrio carrying the same H antigen as *V. cholerae*, appears to be the best procedure for the detection and titration of H antibody of *V. cholerae* in the patient's serum. *V. cholerae* in the form of formalized broth culture failed to be agglutinated by the sera at 37°C. as in type I and in the other types, agglutination of formalized antigen was slower in appearance and was of a lower titre than with a saline live suspension. This indicates that the presence of formalin, more or less completely inhibits O agglutination. Agglutination was, however, quicker and more frequent with formalized antigen at 56°C. than at 37°C. but as we have

seen above, agglutination at 56°C. speaks more in favour of H agglutination than of O agglutination. Therefore, detection and titration of O-antibody with formalized broth culture of *V. cholerae* cannot be recommended.

In type V sera, formalized antigen of *V. cholerae* was agglutinated at 56°C., while that of the non-agglutinating vibrio was not; and in types II and III, the former was agglutinated to a higher titre than the latter. This may apparently suggest that the former is a better antigen for the detection of H antibody but the possibility remains that this better agglutination with the formalized broth culture of *V. cholerae* is due to the O antigen it carries, particularly because of the fact that the saline suspension at 37°C. was agglutinated to the same titre (types II, III and V).

Thus, the use of formalized suspension of *V. cholerae* for detection of either O or H agglutinins in cholera cannot but be deprecated.

Numerous papers have been published on the subject of specific agglutinins in the serum of cholera patients and of contacts, of inoculated persons and of healthy carriers (Pollitzer and Burrows, 1955). Live or killed suspensions have been employed as antigen by some workers, while a formalized broth culture has been used by others and the results have often been most contradictory. Ionesco-Mihaiesi and Cinca (1919) were the first to point out that satisfactory and comparable results can be hoped for, only when live cholera vibrios and not killed ones are used as antigens. Gallut and Brounst (1949) deprecated the use of formalized *V. cholerae* in detecting antibody and recommended those of live suspensions. Yacob and Chaudhri (1945) eliminated some of these possible discrepancies by using live suspensions, but they employed a temperature of 56°C. for two hours. It may be seen from our result that this temperature is not suitable with many cholera sera. Gardner and Venkatraman (1935) appreciated the use of low temperature (37°C.) and short period of incubation (two hours) in agglutination reaction of cholera vibrios, but neither they nor the previous authors went into the best technique for finding out O and H agglutinins of *V. cholerae* in the patient's serum. Pasricha *et al.* (1939) used formalized and living suspension of *V. cholerae* as antigens in studying H and O agglutinins in patient's serum, possibly in the light of Gardner and Venkatraman's (*loc. cit.*) use of the same antigens in raising the corresponding antisera in animals. However, Linton and Seal (1935) showed that boiled suspensions as antigens give much lower titre than living ones. Besides boiling may release non-specific Q antigen of White (1934, 1935). At the same time, it will be observed from our results that formalin does not necessarily completely inhibit O agglutination of *V. cholerae*, so that agglutination with formalized suspension of *V. cholerae* cannot be read as pure H agglutination. The only defect that may possibly accompany the method recommended by us from this study is that the agglutination observed with these antigens may be due to para-agglutinins and co-agglutinins from *E. coli*, *B. alkaligenes fecalis*, *Brucella*, etc. It is wiser to eliminate this possibility by checking slide agglutination of these organisms with the sera. This has been done in our cases and has uniformly been negative. It may, however, be noted that the so-called non-specific agglutination of *V. cholerae* and other enterobacteria have mostly been studied in sera of immunized animals or man and not after natural infection where the possible existence of such Mit-agglutination of Sierakowski (1920) is yet to be proved.

CONCLUSIONS AND SUMMARY.

Fifty samples of sera from bacteriologically positive cases of cholera on the fifth to the seventh day of disease have been studied with a view to find out the best technique for detecting O and H agglutinins of *V. cholerae* in them. Saline suspensions of twenty-four hour agar culture of *V. cholerae* and of non-agglutinating vibrio possessing the same H antigen of *V. cholerae* and formolized broth cultures of the same, have been used as antigens in tube-dilution method, the effect of both temperatures, viz., 37°C. and 56°C., have been studied and readings taken serially up to twenty-two hours. It is concluded that four hours' incubation at 37°C. with live suspension of *V. cholerae* and overnight exposure at 56°C. with formolized broth culture of a non-agglutinating vibrio bearing the same H antigen as the former, offer the best methods for detecting and titrating O and H antibody of *V. cholerae*, respectively.

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EXPERIMENTAL STUDIES ON LIVER INJURY IN MALARIA.

Part I.

PATHOGENESIS.

BY

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IN the past, observations on the pathological processes in malaria had been focussed on changes in the reticulo-endothelial system, particularly in the spleen and the liver. Evidence of parenchyma cell damage was also available from a few autopsy reports on fatal *P. falciparum* cases and the liver injuries observed varied from 'cloudy swelling' and fatty changes to necrosis (Dudgcon and Clarke, 1917; Gaskell and Miller, 1920; Klotz, 1929; De, 1938; Kean and Smith, 1944; Macgraith, 1948). Since systematic investigations on human subjects were not possible, most workers had undertaken studies in *M. mulatta mulatta* (commonly known as rhesus monkeys), experimentally inoculated with *P. knowlesi*, as the behaviouristic pattern of the simian plasmodium has much in common with that of *P. falciparum*. These workers had reported that, besides changes in the cells of the reticulo-endothelial cells, fatty changes and necrosis of the parenchyma cells of the liver were invariably present, particularly at the terminal stage of the infection. Further, while the fatty changes appeared to be of diffuse nature, necrotic processes involved the cells round the central hepatic veins (Taliaferro and Mulligan, 1937; Rigdon and Stratman-Thomas, 1942; Knisely *et al.*, 1947; Macgraith, *loc. cit.*; Ray, 1954; 1955; 1957). It had also been demonstrated that the pathological processes were mild to begin with but became severe, rather abruptly towards the terminal phase of infection, thus indicating some correlation between liver lesions and intensity of infection. Earlier lesions were shown to be swelling with granularity and fatty changes. Subsequently, as the infection progressed, and by the time the parasitic density reached 70 per cent or over, centrilobular necrosis became a dominant feature (Ray, 1954). The necrotic changes appeared to be strictly localized at first around the central hepatic veins but later extended rapidly up to or beyond the midzonal regions. In acute *P. knowlesi* malarial infections, these changes seemed to be the most striking features in the liver

pathology. Other but less conspicuous lesions were the fatty changes which appeared early and seemed to persist throughout. Invariably, changes appeared to be of diffuse nature but in advanced stages they appeared to be more demonstrable near the periportal region (Ray, 1955; 1957a). It had also been observed that these lesions in *P. knowlesi* infection appeared irrespective of the dietary conditions of the host (Ray, 1955).

It has been the general belief that pathological processes, such as swelling with increased granularity, fatty changes and necrosis, were only consequential stages of the 'degenerative changes' caused by the same stimuli. Aetiological factors for initiating such lesions were considered by Maegraith (*loc. cit.*), to be 'stagnant anoxia' (due to retardation of venous outflow on account of the action of a sphincter-like mechanism) as well as a possible 'toxin' of plasmodial origin.

In this context, a recent report on the effect of transfusion and/or choline on the liver, would appear to be of interest. It was demonstrated by Ray (1954a) that transfusion of whole blood or washed erythrocytes from normal monkeys prevented necrosis of the liver parenchyma in *M. mulatta mulatta* infected with *P. knowlesi*, irrespective of the degree of parasitaemia. But fatty changes were still to be observed. Inoculation of plasma from normal monkeys failed to prevent either necrotic or the fatty changes. On the other hand, it was noted that administration of choline in suitable doses minimized the fatty changes to a very great extent although it failed to prevent necrosis. But when choline was administered along with transfusion of whole blood or even washed cells, both the major pathological processes could be prevented altogether.

If one were to consider that both the lesions were the different stages of the same pathological process and that necrosis followed after fatty changes had developed, it would be logical to assume that when the necessary stimuli responsible for bringing about such changes were removed or intercepted, fatty changes would be the first to be prevented. But the experimental evidence did not bear this point out, and as such, the inevitable question arose whether fatty changes and necrosis were not caused by different stimuli. In view of this, Ray (1955) had argued that perhaps anoxaemia was in some way responsible for the centrilobular necrosis, as this could be prevented by combating anoxaemia with transfusion. As to the development of fatty changes in the liver, it was likely that some other factor or factors might be involved, and one of these could well be of the nature of a 'toxin' of parasitic origin, about which repeated references had been made by Maegraith (*loc. cit.*).

The toxin could be of the nature of a foreign protein, such as the parasitic body itself or the metabolites of active plasmodium. The present studies were undertaken to determine the effect of inoculation of killed plasmodia on the liver of normal and anæmic (by bleeding) monkeys. *Pari passu* the role of plasma, from heavily infected monkey on the liver parenchyma of normal and anæmic monkeys, was also explored. A preliminary note on this aspect was published earlier (Ray, 1957a) and Part I of the present communication records the findings of the detailed observations. Results of observations on the pathogenesis of centrilobular necrosis have been recorded in Part II.

MATERIALS AND METHODS.

Host.—Altogether 18 tuberculin negative *M. mulatta mulatta*, weighing between 6 lb. and 10 lb., were utilized for these investigations. Four, weighing between 6 lb. and 7 lb., were used in the first series and 14, weighing between 8.5 lb. and 10 lb., in the subsequent one. Routine 'check up' was carried out in respect of all animals to eliminate any natural infection. Hæmoglobin estimation was carried out initially and subsequently after they had been on standard diet for 3 to 4 weeks.

Diet.—From the time the monkeys were procured and throughout the course of investigations the animals were fed on the standard diet provided to the monkeys maintained at the Malaria Institute of India. Details of such diet have been indicated in the *Appendix*.

Parasite.—A virulent strain (*Nuri*) of *P. knowlesi* (Jaswant Singh, Ray and Nair, 1953) was used for these studies. For inoculation of killed parasites, heparinized blood was drawn from heavily infected monkeys and kept at room temperature for several hours. Just prior to inoculation parasite count was determined. The route chosen was intravenous through the dorsal leg vein and in some cases through the superior mesentric vein as well. For this, laparotomy was done in the usual manner.

Viability test.—Side by side, viability tests were carried out to ensure that the parasites were actually dead. For this, normal monkeys were inoculated intravenously with the killed parasites and blood examination carried out daily. Further, blood from experimental animals was sub-inoculated to normal animals for evidence of development of infection subsequently.

Parasite count was determined with the help of an Ehrlich's eye piece adjusted to count hundred erythrocytes per field of thin film. One hundred such fields were examined and parasites were counted against 10,000 erythrocytes. At least 50 field of thick blood films were examined before entering a negative report.

Stains and staining.—J.S.B. stain (Jaswant Singh and Bhattacharji, 1944) was used for staining thick and thin films in the same slide.

Plasma from infected animals.—Plasma was obtained by centrifugation (in an International refrigerated centrifuge, U.S.A.) of heparinized blood obtained from monkeys heavily infected with *P. knowlesi*. As a rule, the plasma thus obtained was centrifuged again for 2 to 3 times so as to exclude parasites or paritized cells. A dose of 20 c.c. of plasma, mixed with 10 c.c. of physiological saline, was inoculated intravenously through the dorsal leg vein every 3 to 4 hours.

Biopsy.—Liver biopsy was made after laparotomy for which median incision was given under kemithal anæsthesia. 'Geloam' was used as a hæmorrhagic tissue removed were treated in the usual way and paraffin sections were stained with Ehrlich's hæmatoxylin and eosin. For frozen sections a mixture of Sudan III and IV or Sudan black was used.

EXPERIMENTAL RESULTS.

Investigation series I:

General.—As indicated earlier, 4 monkeys were used for these studies. Two of them showed a mean hæmoglobin level of 10.2 g. per 100 c.c. of blood. The two other normal monkeys were made anæmic by repeated bleeding for 5 days till the mean hæmoglobin level reached 5.3 g. against the initial reading of 9.7 g.

All the four animals were then inoculated with killed parasites (*P. knowlesi*). The dose (single) of inoculum for one normal and one anæmic monkey (group I) was calculated to be 4320×10^6 parasitized cells and parasites (a few of the parasites were found to be extracellular) for each of the animals. The route chosen was intravenous through the dorsal leg vein. The other two monkeys (group II, one normal and one anæmic) received each a single dose of 8×10^{10} parasitized cells and parasites through the dorsal leg veins as well as through the superior mesentric vein. As the parasitic infection in the donors chosen was extremely heavy (78 per cent) the volume of blood was not unduly large.

In all cases sub-inoculation tests showed negative results, indicating that the parasites inoculated were actually dead.

Biopsy materials were removed from group I, 3 days after inoculation of dead parasites, while in the other the same was done 5 days after the injection.

HISTOLOGY.

The lobular architecture was normal in all the 4 monkeys. There was no swelling, fatty changes or necrosis of the parenchyma cells in any of the liver sections. The central hepatic veins were normal, so also the portal veins.

However, the sinusoids were found to be dilated in all cases and contained swollen Kupffer cells which were laden with large quantities of yellowish brown or dark brown pigments in granules or in clumps. Besides, cell debris could also be detected in some. The sinusoids also contained large quantities of pigments; normal erythrocytes and a few, what seemed to be, disintegrating parasites. The 'packing' was, however, in no case as severe as seen in the liver sections obtained from monkeys with severe *P. knowlesi* infection, as described earlier by Ray (1957).

Investigation series II.—The studies were undertaken in 12 normal and 2 anæmic (made so by repeated bleeding) monkeys. Out of 12 normal animals two received plasma obtained from normal monkeys and they served as the control group. Under the experimental group, seven normal animals were placed under group I and were treated with plasma obtained from donor monkeys showing heavy *P. knowlesi* infection. The remaining 3 normal monkeys received choline as well as plasma from infected animals (group II). Plasma from infected monkey was also administered to the 2 anæmic monkeys (group III). For convenience, the disposition of the animals is indicated as follows:—

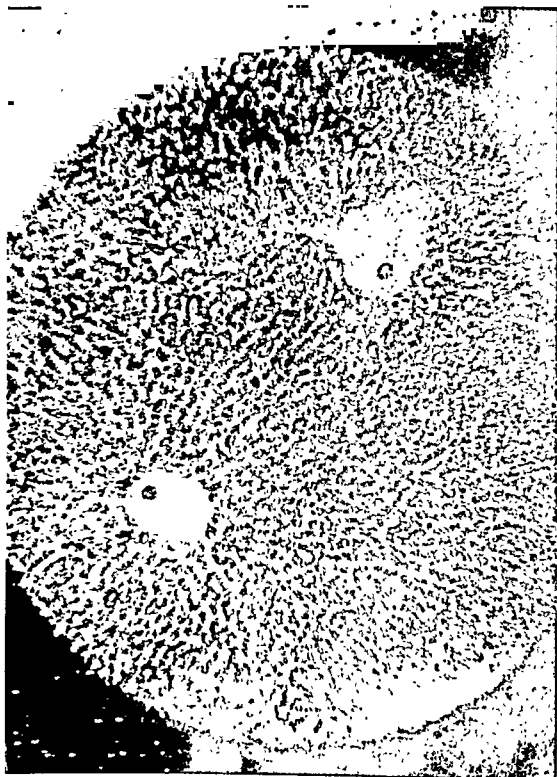


FIG. 1. Frozen section of the liver stained with Sudan black shows extensive nature of the fatty changes though such changes are more pronounced in the periportal region (low power).

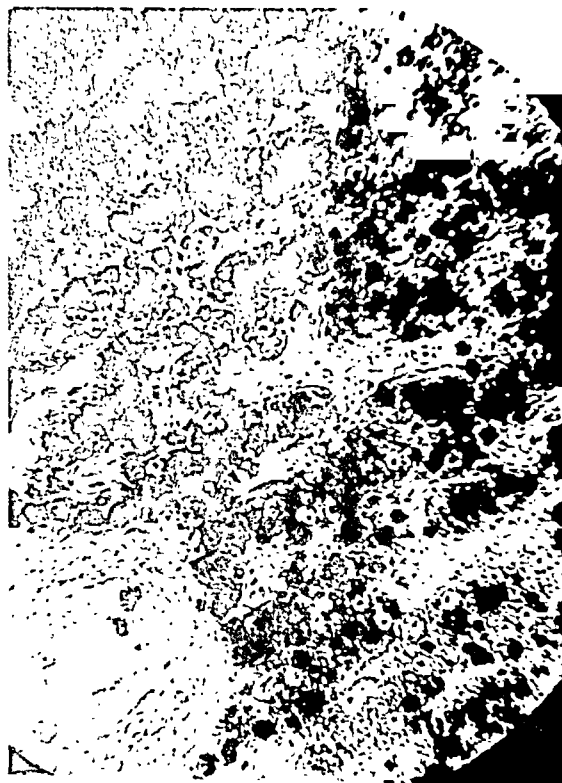


FIG. 2. Frozen section of liver stained with Sudan black showing pronounced fatty changes round the portal tract (same section under high power).

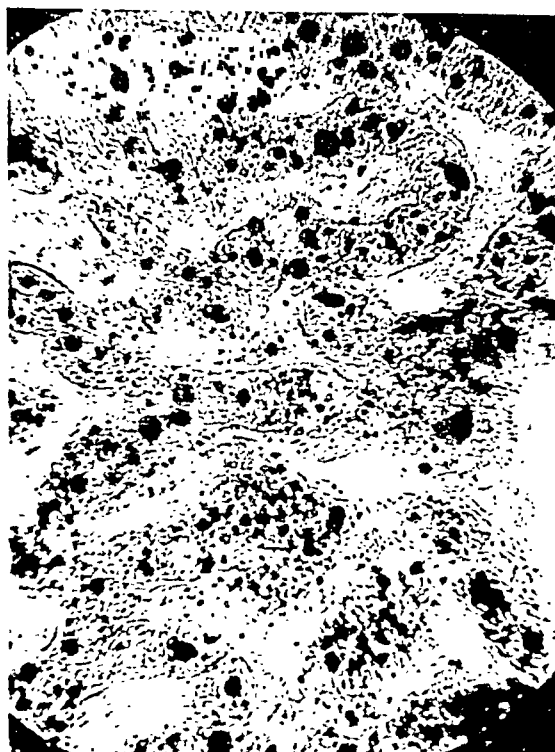


FIG. 3. Frozen section of liver stained with Sudan black shows the disposition of fatty changes round the central hepatic veins (same section under high power).

	Number of monkeys.	Inoculation of plasma from.	Any other treatment.
A. Control group	2	Normal monkeys	..
B. Experimental group			
Group I	7	Heavily infected monkeys	..
Group II	3	" " "	Choline
Group III	2 (anaemic)	" " "	..

The amount of plasma (obtained either from normal or infected animals) was 20 c.c. for each monkey, diluted with normal saline and administered intravenously every 4 hours for a period of 72 to 96 hours. The dose of choline for the monkeys under group II was 60 mg./kg., administered orally twice a day during the period when plasma from infected animals was injected and also for one day prior to such inoculation.

In all cases, the first biopsies were made 12 to 24 hours after the last inoculation and also in most cases for the second time after 4 to 7 days.

PATHOLOGICAL CHANGES.

Control group.—The liver lobules retained the normal architecture. The parenchyma cells were normal, so also the central hepatic veins and the sinusoids. The portal veins did not show any changes either.

Experimental:

Group I.—(a) Liver sections, obtained soon after inoculation of the last dose of infected plasma, showed the following pathological changes:

The lobular architecture was well preserved. The parenchyma showed some degree of swelling but the most conspicuous feature was the enlargement of the cells. Under the low power, the distribution of fat droplets (as seen in frozen sections treated with Sudan stains) appeared to be somewhat more concentrated round the central hepatic veins (Plate XIII, fig. 1). On closer study under high power, it was found that the degree of fatty changes was much heavier and the droplets were more numerous in the periportal region than round the central hepatic veins (Plate XII, fig. 2). In the latter area such changes were either absent as observed in some specimens (Plate XIII, fig. 3). There were no necrotic changes in any of the specimens. The central hepatic veins were normal.

The sinusoids appeared to be somewhat narrowed on account of the swelling or fatty changes in the parenchyma cells. The endothelial cells (littoral cells) appeared to be flattened as the sinusoids also retained the normal appearance.

(b) Biopsy materials, obtained from 6 out of 7 monkeys, were examined the first biopsy 4 to 7 days after the last inoculation of infected plasma.

more or less normal structure of lobules and the parenchyma cells. The extensive fatty changes observed after the first biopsy were altogether absent. The central hepatic veins and other structures were found to be normal.

Group II.—The lobular structure was normal. Only minor degree of swelling with some granularity of the parenchyma cells was detectable in most of the lobules. The fatty changes were either absent altogether or when present were hardly conspicuous. There was no evidence of necrosis. The central hepatic veins and other structures appeared to be normal.

Group III.—The liver lobules retained the normal architecture. However, there was some swelling of the cells and extensive fatty changes, particularly well marked in the periportal region. The fat droplets were much larger than those seen near the centre of the lobule. No necrosis could be detected in any of the specimens.

The central hepatic veins appeared to be normal. The sinusoids appeared to be somewhat narrowed. The portal veins retained normal appearance.

DISCUSSION.

Anderson and Morrison (1942) were of the opinion that lesions in simian malaria were caused by anoxæmia due to vascular occlusion, associated with severe anæmia. The development of histo-toxic anoxia in malaria was also considered by Hartman (1937) and Rigdon (1942). Subsequently, Maegraith *et al.* (1947) reported that the liver changes, such as centrilobular necrosis, were due to stagnant anoxia caused by retardation of venous outflow on account of the action of a sphincter-like mechanism in the hepatic venous tree. Although this is the generally accepted view to-day, Maegraith (*loc. cit.*) had not over-ruled altogether the possible role of some toxic factor, as he believed that 'at this stage in our knowledge, however, it is not possible to rule out direct toxic effects on the cells and the search for possible toxic agents must be continued'. Although during the current series of investigations some explanations in the pathogenesis of centrilobular necrosis had been furnished by Ray (1954; 1954a; 1955), Ray and Sharma (1957), it was realized that there were still considerable lacunæ in our knowledge of the subject which required detailed studies, particularly on the role of malarial toxin, if any.

It had been observed by many workers that malarial paroxysm, or in fact the whole clinical picture, indicated the introduction of some non-specific agents and that probably they were liberated in the plasma at the time of sporulation. According to Meleney (1941) and Viswanathan (1944) perhaps some specific soluble product of the parasite or a byproduct of its metabolism might be involved. Although, Brown (1912) believed that the malarial pigment, hæmozoin was a potential toxic substance, later workers, such as Morrison and Anderson (1942), did not consider it to be responsible for malarial paroxysm or lesions. Malarial paroxysm and liver lesions were attributed to other products of sporogony as well, such as foreign protein, cell debris, certain metabolic products of the parasite such as pyruvic acid, etc., and some of these could either be of parasitic or of endogenous origin (Massias, 1948; Maegraith, *loc. cit.*). Further, Gear (1946) believed that there was a possibility of alteration in the chemical structure of the cells, altered by malaria or anoxia in such a way that the tissue became

antigenic to itself and consequently suffered injury. Thus, it would seem that the nature of toxin, if any, its effect on the cells or the mode of action, are still a matter of conjecture.

In view of these, the present investigations would appear to be of interest as they throw some light on the subject of 'toxin' as well as its relation to the pathogenesis of liver injuries. It was demonstrated that introduction of massive doses of killed parasites failed to produce significant lesion in the parenchyma cells. Even in the series in which the parasites were introduced through the superior mesentric veins to anæmic monkeys, thereby making conditions suitable for the development of liver injuries, no hepatic lesion could be detected. Since no parenchyma damage could be observed, it is reasonable to assume that the parasitic bodies do not exert any damaging influence over the liver cells. In that even, it is perhaps likely that some metabolic product of the plasmodium is possibly involved. Since in many cases of black-water fever, liver lesions were observed even in spite of very scanty infection, Macgraith (*loc. cit.*) indicated that perhaps a diffusible substance, produced directly or indirectly during the course of the disease, was involved in the pathogenesis of liver injury of this type.

Were we to accept this concept, it may be argued that this particular metabolic product is specific in respect of *P. knowlesi* and is not elaborated by *P. cynomolgi* as there is no significant liver lesion due to the latter type of plasmodial infection (Taliaferro and Mulligan, *loc. cit.*; Ray, 1955). Although no necrotic changes were evidenced, swelling of the cells and fatty changes in the liver of normal monkeys were invariably present shortly after the last dose of plasma inoculation. But there were no such changes in normal monkeys receiving plasma from normal animals. Further, the distribution of fatty changes was also highly interesting. In *P. knowlesi* infection, it had been observed earlier that fatty changes were of diffuse nature in most cases (except in some where they appeared to be periportal when necrosis was well advanced), and thus making it difficult to locate the origin (Ray, 1957).

In the present series, it was noted that the fatty changes were quite heavy round the portal tracts as compared to the centrilobular zone. Further, the fat droplets were found to be larger in size in the periportal region. In the normal course of events the flow of inoculated plasma through the liver would be from the portal vein towards the central vein through the sinusoids. Therefore, were there any toxic elements in the plasma obtained from infected monkeys and were they capable of inflicting injury to the liver cells, obviously the cells nearer the portal tracts would be the first to bear the major brunt. This seemed to have actually happened as the fatty changes were more conspicuous in the periportal than the centrilobular area. As the changes were of reversible nature (after a few days), it may be inferred that the toxin had either been excreted out or neutralized during this period.

The liver changes in anæmic monkeys after inoculation of plasma (infected) can be well understood as the parenchyma cells were already in a state of partial anoxia and thus susceptible to injury.

From the above observations one is inclined to believe that the plasma from infected animals contained certain factor or factors which were responsible for the

development of fatty changes in the liver. Further, it was noted that lipotropic agents, such as choline, were able to prevent or minimize such fatty changes when choline was administered along with plasma. In this connection, attention may be drawn to the earlier findings of Ray (1954a) who had demonstrated earlier that while transfusion of whole blood or erythrocytes prevented or reversed necrosis, fatty changes could be minimized by choline, though by itself choline was unable to prevent necrosis.

These investigations and those observed by Ray and Sharma (*loc. cit.*) would indicate that while liver changes, such as necrosis, was perhaps due to a complicated mechanism, fatty changes were caused by certain elements present in the plasma of infected animals. But in the presence of choline the injurious effects of plasma seemed to be neutralized.

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APPENDIX.

Diet provided to the experimental monkeys.

Ingredients.	Quantity*, oz.	Fat, g.	Protein, g.	Carbohydrate, g.	Cal. value.
Gram ..	3 to 4	4.5-6.0	14.7-19.6	52.2-69.6	270-360
Wheat flour ..	3 to 4	1.5-2.0	10.2-13.6	61.5-82.0	300-400
Vegetables or Fruits: ..	4†	0.4	1.6	16.4	76
(a) Guava or ..					
(b) Sweet potato..		0.4	1.2	35.2	148
Total ..		6.4 to 8.4	26.1-26.5 to 34.4-34.8	130.1-148.9 to 166.0-186.8	646-718 to 836-908

*The quantity in oz. varied according to the weight of the monkey. Monkeys weighing between 8 lb. and 12 lb. received 4 oz. of each, while those weighing below 8 lb. received 3 oz. of gram and wheat flour (in the form of chappatis), and 4 oz. of vegetables or fruits.

†Item varied according to the season of the year.

Ind. Jour. Med. Res., 46, 3, May, 1958.

EXPERIMENTAL STUDIES ON LIVER INJURY IN MALARIA.

Part II.

PATHOGENESIS.

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In part I of this communication, it had been indicated that the liver parenchyma damages in fatal *P. falciparum* infection in man and *P. knowlesi* in *M. mulatta mulatta* were of the nature of swelling, fatty changes and centrilobular necrosis.

Working with the latter plasmodium, Ray (1954; 1955; 1957), had indicated that there was some correlation between liver lesion and the intensity of infection, particularly in respect of the necrotic changes. Such changes appeared to develop when the cell infection had attained between 40 and 50 per cent and at this stage the lesion was usually limited to the cells round the central hepatic veins. But soon after, the lesion had spread rapidly up to or beyond the midzonal region and this seemed to coincide with the terminal phase of the infection. Besides these, another interesting feature was the dilatation and sometimes even rupture of the central hepatic veins, particularly when the necrotic changes were well advanced.

The sinusoids were often widely dilated and contained enormously swollen Kupffer cells, parasitized cells, pigments, cells debris and blood elements and these 'packings' would appear to form a mechanical blockage to the free flow of blood from portal to hepatic veins (Ray, 1955). The portal veins were found to be either normal or as in some cases somewhat dilated.

In the absence of definite knowledge pathogenesis of liver injuries of the type mentioned has remained a matter of speculation. However, the recent hypothesis as postulated by Maegraith (1948) that the lesions were due to 'stagnant anoxia' on account of constriction of a sphincter-like mechanism in the hepatic-venous tree and that a humoral or reflex mechanism was perhaps involved in setting up the chain reaction, has focussed the attention of many workers, though the *modus operandi*, nature of stimuli or the mode of transmission of such stimuli still remain obscure.

Meanwhile, it had been demonstrated that transfusion of whole blood or washed cells from normal monkeys prevented necrosis but not fatty changes in *P. knowlesi* infection, whereas choline in suitable doses minimized fatty changes but failed to prevent necrosis (Ray, 1954a). Further, it was also shown that plasma from infected animals, when inoculated to normal monkeys, caused extensive fatty changes and these could be prevented by concurrent administration of choline (Ray, 1957a). These findings would, therefore, lend support to the belief that the two pathological processes, such as fatty changes and centrilobular necrosis, were perhaps initiated by different stimuli. During previous observations, Ray (1955; 1957) had indicated that so far as the necrotic changes were concerned, there were indirect evidences of the existence of some constrictor mechanism in the hepatic-venous tree and it was likely that it was thrown into action by some stimuli originating in some higher centres and transmitted through the sympathetic chain. It is well known that sympathetic action constricts most blood vessels, especially of the splanchnic viscera (Brain, 1951). It was also hinted that the factor which stimulated such higher centres could be anoxæmia.

In order to elucidate this point further, investigations were undertaken to observe the effect of *P. knowlesi* infection on the liver of monkeys previously subjected to sympathectomy as compared to the results in another group of infected animals in which such operation was not undertaken. A report on the preliminary findings had already been communicated earlier (Ray and Sharma, 1957) and the present paper records the detailed observations on the subject.

MATERIALS AND METHODS.

Materials.—Twenty two *M. mulatta mulatta*, weighing between 10 lb. and 12 lb., were utilized for these studies. All were subjected to tuberculin tests (Nair and Ray, 1954) and found negative. Prior to the investigations they were kept on standard diet as indicated in Part I of this paper, and body-weight checked up periodically. Twelve of these animals which formed the experimental group were subjected to sympathectomy, while 10 others were kept as the control group. Subsequently, both the groups received a standard dose of parasites. But before inoculation it was ensured that the wound was completely healed up and leucocyte count was normal in those subjected to the operation. This was to eliminate any infective condition. Normally, a monkey was ready for such investigation in the the course of 4 to 6 weeks. Adequate diet was provided and measures taken for the prevention of diarrhoea or dysentery.

Parasites.—A recently isolated strain (Nuri) of *P. knowlesi* (Jaswant Singh, Ray and Nair, 1953; Edeson and Davey, 1953) was used for these studies. The strain is highly virulent and irrespective of the dose of inoculum or the route, the course of infection is rapidly progressive once patent infection is established. The density of parasites may reach even up to 96 per cent cell infection prior to death though in the majority of cases the range varies between 60 and 80 per cent, accompanied by a very rapid fall in the hæmoglobin level (Ray, 1955). For experimental inoculation during the present series of investigations, a challenging dose of 5×10^6 parasitized cells per kg. was introduced intravenously to monkeys belonging to both the groups of experimental and comparative series. The dose had been standardized earlier in the Laboratories of this Institute for various types of investigations. With this inoculum, patency is established within a few hours, and the monkeys usually succumb to the infection between 4 and 6 days.

Stains and staining.—Blood smears were stained with J.S.B. (Jaswant Singh and Bhattacharji, 1944; Jaswant Singh, Ray and Nair, 1953). An Ehrlich's eye piece adjusted to include 100 erythrocytes per field of thin film was used for parasite count. One hundred such fields were examined.

Sympathectomy.—According to Kuntz (1933) sympathetic supply to the liver in *M. mulatta* (rhesus monkeys) comes from coeliac plexus which derives its main fibres from the 6th to 12th thoracic ganglions. In view of this, sympathetic chain was removed in 12 monkeys at varying levels from the 6th to 12th thoracic region. The anæsthetic used was intravenous Kemithal (I.C.I. preparation) the use of which had been previously standardized at this Institute for all major operations on monkeys (Jaswant Singh, Ray and Nair, 1951).

The operation sympathectomy consisted of a $3\frac{1}{2}$ " horizontal incision over the corresponding rib (depending on the level of resection required) on the right side extending from paravertibral line to anterior auxilliary line. The posterior third of the rib was resected. The pleura was stripped from the inner surface from the adjacent ribs and vertebral bodies. The pleura and the lungs were retracted medially. The sympathetic chain was identified and exposed carefully over two to three segments above and below the level of incision. The corresponding ganglions at the particular levels required to be removed were identified and the sympathetic trunk along with the ganglions was removed after cutting the

rami communicantes of each ganglion. Materials obtained at operation were subsequently subjected to histological studies. The wound was closed layer by layer. After a lapse of 10 days to a fortnight, operation was carried out on the other side in all but one.

Out of the 12 experimental monkeys, one was subjected to unilateral sympathectomy (group I) and seven bilateral (group II) at the levels between 6th and 8th thoracic segments. In two other groups consisting of 2 animals in each, similar operations (bilateral) were carried out at 8th to 10th (group III) and 10th to 12th (group IV) segments, respectively.

During the post-operative period each monkey received 100,000 unit procain penicillin per 10 lb. body-weight for 4 to 5 consecutive days. The standard diet (*Appendix* of Part I of this communication) was supplemented with milk during this period.

Preparation of tissues.—Liver tissue was removed at the terminal stage of infection just prior to the death of monkeys. The materials were fixed in formol saline and imbedded and sectioned in paraffin in the usual manner. Ehrlich's acid hæmatoxylin and eosin were used for routine staining. For demonstration of fat, frozen sections were cut and stained with a mixture of Sudan III and IV or Sudan black.

RESULTS.

A. Parasite density:

The intensity of infection as determined by parasite density in the control and the experimental series was as follows:—

(i) *Control series.*—The parasite count in ten monkeys varied from 52 to 86 per cent cell infection, the mean density being 68 per cent.

(ii) *Experimental series.*—The parasite density has been shown groupwise in the Table:—

TABLE.

Group.	Number of animals.	PARASITE DENSITY EXPRESSED AS PERCENTAGE OF CELL INFECTION:	
		Range.	Mean.
I	1	..	61
II	7	46 to 90	72
III	2	58 to 72	65
IV	2	48 to 57	52·5

The degree of mean parasitæmia in animals of the control series and those in groups I to III in the experimental series was somewhat similar. In group IV the parasitæmia was somewhat less. This difference was mainly due to small number of experimental subjects. However, it was still well within the range and had attained a level at which normally necrosis of the cells round the central hepatic vein is invariably present in majority of the liver lobules as indicated earlier (Ray, 1957).

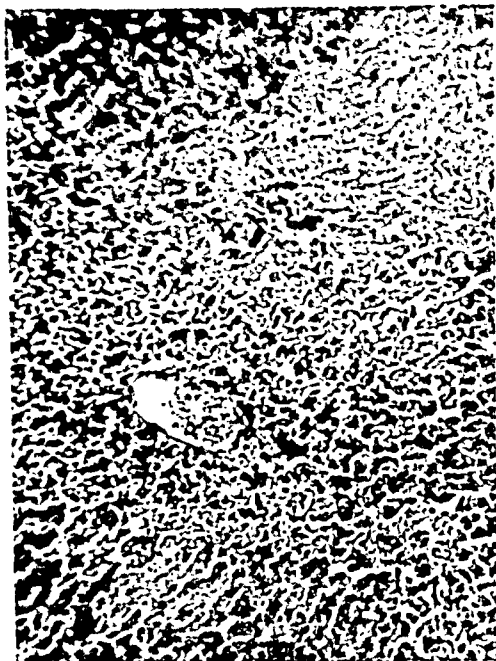


FIG. 1. Paraffin section of liver stained with H. E. shows centrilobular necrosis in *P. knowlesi* infection.

FIG. 2. Paraffin section of liver stained with H. E. showing fatty changes and necrosis of the parenchyma cells: also dilatation and rupture of the central hepatic veins in *P. knowlesi* infection.

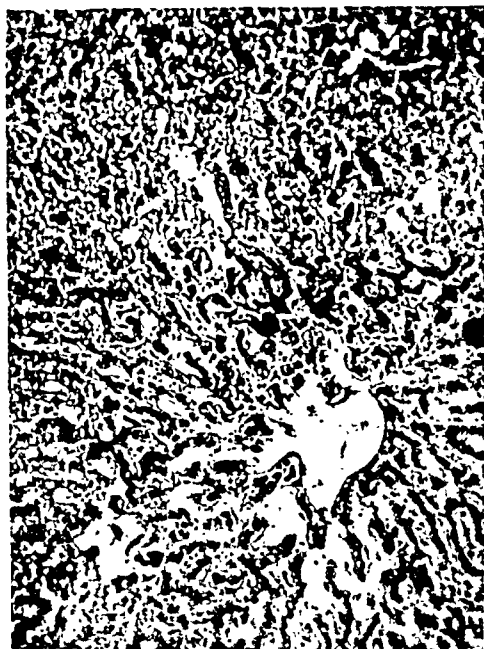
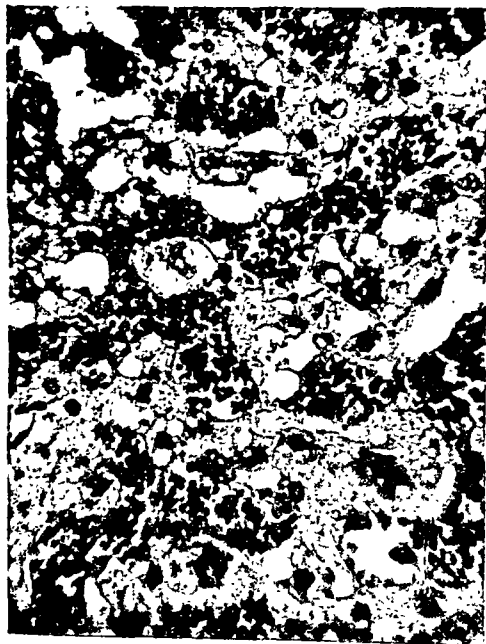


FIG. 3. Paraffin section of liver stained with H. E. showing heavy sinusoidal 'packings' in *P. knowlesi* infection.

transfusion or after bilateral sympathectomy, i.e., under either of the two apparently uncorrelated approaches. In view of this, it would be necessary to discuss fully the aetiological factors responsible for liver changes in fatal malarial infections.

Knisley *et al.* (1941) believed that the centrilobular necrosis of the liver was due perhaps to the mechanical obstruction of the free flow of blood through the sinusoids from the portal to central hepatic veins. The blockage might be caused by swollen Kupffer cells, accumulation of free phagocytes, cell debris, parasites, parasitized cells, pigments or masses of red cells in a state of 'sludge' or thrombus. From the nature of 'packing' in the sinusoids observed in the liver sections during the present studies, one would normally be tempted to uphold the mechanical obstruction theory, whether it is complete or partial. It is well known that as it is, under normal conditions, the cells near the centre of the lobules are at a lower oxygen tension than those in the periportal region. Any obstruction even be it partial, would slow down the free flow of blood and, therefore, the cells farthest from the portal tracts would suffer the most.

In view of the above, it is felt that attention may again be invited to two important observations; dilatation or rupture of the central hepatic veins in un-interfered *P. knowlesi* infection and the prevention of necrotic changes by transfusion of whole blood or washed erythrocytes as indicated earlier. Were the 'blockage theory' alone given the credence, it would be difficult to reconcile to the above facts. Since the sinusoids were found heavily packed, one would expect that the flow of the transfused blood would also be retarded. This would, therefore, result in lowering of the oxygen tension of the blood or washed erythrocytes by the time they reached the central hepatic veins in the same way as happens with the normal flow as already explained. Thus, even after transfusion one would expect necrosis of the cells round the central hepatic veins but which did not occur. This particular point would be discussed further at a later stage. Again a blockage of the sinusoids would lead to collapse of the central hepatic veins rather than dilation or rupture. But were there any retardation of the outflow from the central hepatic vein itself on account of some kind of obstruction higher up, dilation or even rupture of the vein would be quite conceivable. In that event, necrotic changes would be the result of stagnant anoxia. In this connection, it may be observed that Rigdon and Stratman-Thomas (1942) had reported that the centrilobular necrosis in fatal infection of *P. knowlesi* was secondary to congestion following cardiac failure. Myocardial failure was the result of severe anaemia and led to 'back pressure'. This, in turn, was responsible for necrosis. But various workers, including Maegraith (1944), failed to observe cardiac failure in fatal human malaria though liver injury, including necrosis, was found to be extensive. In view of this, he considered that back pressure 'cannot have any part in producing liver lesions'. Further, it may be noted that in *P. cynomolgi* infection the degree of anaemia developed could be even of more severe type than that observed in *P. knowlesi* infection (Ray, 1957b) and yet no necrotic changes in the former case were detectable (Talliaferro and Mulligan, 1937; Ray 1955). There were also no necrotic changes in monkeys rendered anaemic by repeated or sudden withdrawal of blood (Ray, 1955).

On the basis of their detailed studies on the subject, Maegraith and Findley (1944) and Maegraith (1948) believed that neither 'back pressure' from failing

heart nor mechanical obstruction of the sinusoids could possibly be responsible for such liver lesions as observed in malaria. On the other hand, these workers considered that active obstruction to the hepatic venous outflow would appear to be the most reasonable interpretation. Such obstruction could perhaps be due to the existence and operation of a possible sphincter in the hepatic venous tree for the activation of which humoral or some reflex mechanism might be involved (Maegraith *et al.*, 1947; Maegraith, 1948; and Maegraith *et al.*, 1949). There is already some evidence to show the existence of such mechanism in the liver of frogs and also in dogs (Bauer *et al.*, 1932; Knisley, 1939). Although no sphincter has yet been clearly demonstrated in man or monkeys, Maegraith (1948) believed that a similar effective mechanism could very well be present in man as well.

As to the changes in the parenchyma cells as shown during the present series of investigations, it was observed that in *P. knowlesi* infections in *M. mulatta mulatta* (not subjected to sympathectomy) there was severe degree of necrosis in the cells round the central hepatic veins, particularly during the terminal phase. Similar lesion was also observed in the monkeys previously subjected to unilateral sympathectomy. Such centrilobular type of necrosis had also been described earlier in acute syndrome of Chiari's disease (Chiari, 1899) and also by Simonds (1935) in subjects with ligation of the inferior vena cava. Later, Hill (1951) demonstrated the same in venous occlusive disease in which there is obstruction to the venous outflow on account of sub-endothelial proliferation of the hepatic veins. Further, it may be mentioned that occlusion of the hepatic veins due to thrombosis with organization as seen in Senecio poisoning also produces centrilobular necrosis (Sapeika, 1952). Liver injuries, such as centrilobular necrosis produced under the above-mentioned conditions, have great degrees of similarities to those produced under *P. knowlesi* infection. The findings, such as dilation or rupture of the central hepatic veins, congestion and dilatation of the sinusoids and finally centrilobular necrosis, in unsympathectomized monkey would all point out to the theory of retardation of venous outflow perhaps on account of some constrictor mechanism in the hepatic venous tree as was conceived by Maegraith *et al.* (1948).

In view of the above, the observations, such as complete absence of necrotic changes and the almost normal condition of the central hepatic veins in the liver of monkeys previously subjected to sympathectomy, would appear to be highly significant. Because, this would furnish further evidence to the existence of a sphincter mechanism which is perhaps operated by stimuli originated at some higher centre, possibly in the hypothalamic region and transmitted through the sympathetic fibres supplying the liver. Were this conception to be finally accepted, it would be necessary to establish the existence of a sphincter mechanism (perhaps by careful and systematic serial sections of the liver), the higher centre itself and also the factor or factors responsible for activating such a centre. Studies on the above aspects are already in progress.

Since transfusion of whole blood or washed erythrocytes prevented necrotic changes in spite of sinusoidal blockage, as mentioned earlier, Ray (1955) has argued that transfusion had merely helped to combat the initial anoxæmia caused by destruction of large number of erythrocytes in *P. knowlesi* infection, and that anoxæmia would perhaps be responsible, in some way, in setting the mechanism

into operation. In the light of the present findings, one would be tempted to draw the inference that perhaps anoxaemia was the stimulant to the hypothalamus.

But, before one could come to a final conclusion, it would be necessary to explore also the role of 'toxin' (if any) about which repeated mention had been made by Macgrath (1948). That the factor/factors involved could produce fatty changes and not necrosis, has already been demonstrated in Part I of this communication.

It is thus felt that the present studies have thrown open some newer channels of investigations on this subject.

SUMMARY.

1. Bilateral sympathectomy between 6th and 12th thoracic level was carried out in 11 *M. mulatta mulatta*. One monkey was subjected to similar operation but on one side only. Four to six weeks later they were inoculated with a standard dose of 5×10^6 parasitized cells from donor monkeys infected with *P. knowlesi*. Ten other normal monkeys were similarly infected and served as the control series.

2. The course of infection and overall mean density of parasitaemia in both the series appeared to be more or less similar and all animals died between 4 and 6 days.

3. Liver biopsies were made just prior to death. In the control series and in the monkey subjected to unilateral sympathectomy the liver lesions were significant and seemed to be proportional to the degree of infection. Fatty changes and necrosis of the parenchyma cells were the main features. The necrotic changes were restricted in the cells round the central hepatic veins which were often dilated and in certain advanced cases ruptured. In those in which parasitaemia was high the necrosis had spread up to or beyond the midzonal area. The origin of fatty changes could not be located in many cases but where necrosis was found to be severe, these appeared to be of periportal distribution. In contrast, none of the monkeys subjected to bilateral sympathectomy showed any significant parenchyma damage. The central hepatic veins appeared to be normal.

4. The sinusoidal changes and the 'packings' with swollen Kupffer cells, pigments, parasitized cells, erythrocytes, etc., appeared to be more or less similar in all monkeys belonging to the experimental or control series.

5. The possible logical course of events was discussed and it seemed likely that a sphincter mechanism in the hepatic venous tree was operative due to some stimuli originating perhaps in the hypothalamic region and transmitted through sympathetic chain. The nature of stimulus could very well be anoxaemic. However, the rôle of a possible toxin needed further investigations.

6. The present work, therefore, would appear to give a lead to further studies on the pathogenesis of liver injury.

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AN ELECTROPHORETIC STUDY OF PROTEIN PATTERNS OF SERUM FROM A FEW SPECIES.

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ELECTROPHORESIS as a method of separation of chemical constituents has proved valuable in many analytical studies. The qualitative as well as quantitative aspects of plasma in health and disease have been worked out in detail by various workers. But only a few references are available in the literature regarding the utility of this method in investigating the comparative patterns of plasma proteins in various species. This communication is a preliminary report on a study undertaken to find out whether electrophoresis can be employed to differentiate the patterns of plasma proteins in different species. The present paper describes the results of our study of the patterns of the serum proteins in man, cow, goat and rat.

EXPERIMENTAL MATERIAL AND METHOD.

The source of human blood samples was the student population of this College. Blood samples of cows and goats were obtained from the local slaughter house and those of rats by bleeding under anæsthesia from the tail vein of albino rats reared in the Department. Attempts were made to reduce hæmolysis to a minimum. The blood was collected in a perfectly dry test-tube and after clotting was centrifuged for five minutes at 3,000 r.p.m. The serum thus obtained was used for the study.

An apparatus supplied by Messrs. A. H. Thomas (serial No. 155) was used for electrophoresis. All the analyses were made in a barbital buffer at pH 8.6 and 0.05μ . The composition of the buffer was 10.3 g. of sodium diethyl barbiturate and 1.84 g. of diethyl barbituric acid dissolved in 1 litre of distilled water.

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The electrophoretic cabinet was kept in a refrigerator so as to maintain a constant temperature of 4°C. Filter-paper strips (Whatman No. 1, size 30.5 × 2.5 cm.) were stretched taut on a bakelite frame and kept in the cell for equilibration. After equilibration the bakelite frame was taken out and small paper strips soaked in serum samples carrying a load of about 0.01 c.c. to 0.02 c.c. were applied over the paper strips on the frame. The separation of the proteins was obtained by passing a current of 1 m.a. at 250 Volts D.C. per paper strip for 8 to 10 hours. Then the strips were taken out and dried for 10 minutes at 100°C. The dried strips were dyed in amidoblack and then washed successively in a clearing fluid and tap-water until a pale blue background was left. The papers were then dried and scanned using a densitometer (Photovolt Corporation).

When scanning, readings were taken at intervals of 1 mm., starting from the base line and going through the whole pattern. Two readings were taken at each of the points by setting the amplifier needle at zero on two points of the strip—one to the left and the other to the right of the pattern. The average of the two readings was taken to be the optical density at a particular point.

Graphs were drawn and divided into sections on the basis of major peaks and troughs. Any graph which did not permit of division into at least four sections corresponding to α -, β -, and γ -globulins and albumin was rejected. One or two samples had to be rejected due to hæmolysis which made the β -section unduly predominant. Only in the case of human sera could α_1 - and α_2 -globulins be differentiated on the graphs and these were added together to give an α -section. There were altogether 87 samples—24 of human sera, 20 of cow's sera, 21 of goat's sera and 22 of rat's sera. Thirteen of these (4, 3, 5 and 1, respectively, from the 4 species) had to be rejected for the reasons given above.

For each section, area was calculated by means of Simpson's one-third rule for numerical integration which is as follows: If an interval $b-a$ is divided into $2n$ sections of width $h = \frac{b-a}{2n}$ and if Y_i is the observation at $a + ih$, then the area over the interval is given by the expression:

$$\frac{h}{3} [(Y_0 + Y_{2n}) + 4(Y_1 + Y_3 + \dots + Y_{2n-1}) + 2(Y_2 + Y_4 + \dots + Y_{2n-2})]$$

Here, h was taken to be 1 mm. In some sections there were not an odd number of observations. In that case the last observation was set aside and the above formula was used. To the value thus obtained the average of the last and the last but one observation was added to get the area for the whole section.

For each sample, the areas of the four sections were expressed as percentages of the total area for that sample. These percentages correspond to the percentages of the corresponding proteins in the serum of that individual.

Per cent compositions.—A summary of the electrophoretic partitions of the samples of sera from the four species is given in Table I.

The number of samples in each species, mean values of the percentages of the proteins with estimates of standard deviations and coefficients of variations are given in Table I. It is seen that human sera have more of albumin and less of β -globulin compared to the other species. Rat's sera have got more of β -globulin, but not differing by a large amount from the other species.

TABLE I.
Electrophoretic fractionation of serum proteins of four species.

Species.			COMPOSITION, PER CENT.				Number of samples.
			γ -globulin.	β -globulin.	α -globulin.	Albumin.	
Man	Mean	..	23.51	14.38	13.74	48.37	20
	S.D.	..	2.58	2.98	2.17	2.25	
	C.V.	..	10.97	20.74	15.76	4.65	
Cow	Mean	..	23.11	20.53	15.55	40.81	17
	S.D.	..	6.16	4.87	3.64	3.27	
	C.V.	..	26.68	23.72	23.38	8.02	
Goat	Mean	..	26.64	20.55	10.09	42.73	16
	S.D.	..	6.52	4.70	2.52	5.11	
	C.V.	..	24.48	22.89	25.00	11.96	
Rat	Mean	..	23.08	25.66	10.06	41.20	21
	S.D.	..	4.00	3.05	2.37	5.40	
	C.V.	..	17.35	11.87	23.56	13.11	

Note.—Mean = $\bar{x} = \sum x / N$, where x is the observed percentage for an individual and N is the number of samples in the species.

S.D. = estimated standard deviation = $\sqrt{\sum \frac{(x - \bar{x})^2}{N-1}}$

C.V. = estimated coefficient of variation = S.D./mean.

It is interesting to note that the C.V. are much higher in the case of cow and goat than those for man and rat. In this connection, it may be remembered that the blood samples for the two species were obtained from the local slaughter-house. Moore (1945) has also noted a somewhat similar effect. He obtained values for swine serum and it was found that the values obtained from a sample from a New York city slaughter-house were much different from those obtained from 3 swines raised privately. Since, there was only one sample from the slaughter-house, he could not, of course, compare the C.V. between the two cases.

Typical patterns, one for each species, are shown in the Text-figure.

Lower and upper confidence limits for means and upper confidence limit for S.D. have been calculated and are given in Table II:—

TABLE II.
Estimated confidence limits for means and S.D. of Table I.
(Confidence level 95 per cent.)

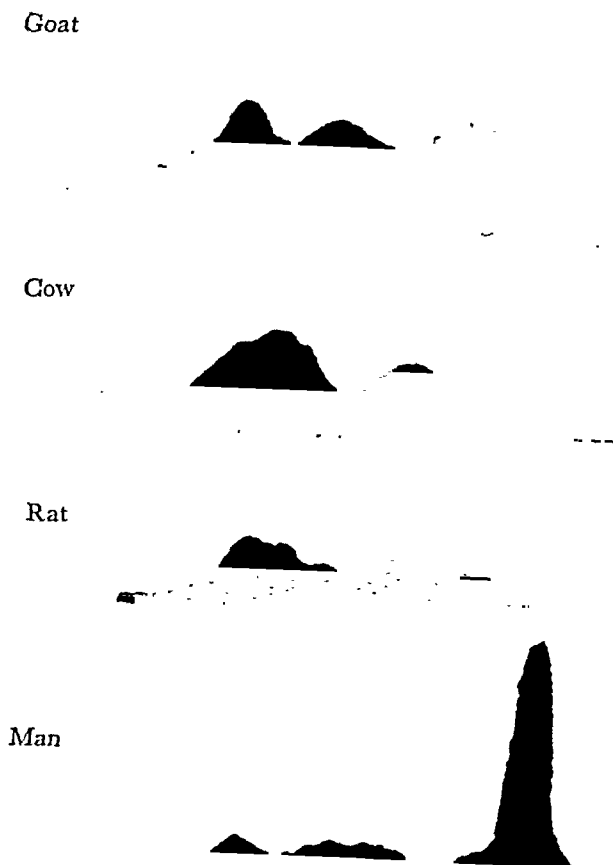
Species.	γ -GLOBULIN.			β -GLOBULIN.			α -GLOBULIN.			ALBUMIN		
	Mean.		S.D.	Mean.		S.D.	Mean.		S.D.	Mean.		S.D.
	L.L.	U.L.	U.L.	L.L.	U.L.	U.L.	L.L.	U.L.	U.L.	L.L.	U.L.	U.L.
Man ..	22.30	24.72	3.88	12.98	16.17	4.49	12.73	14.75	3.26	47.32	49.42	3.39
Cow ..	19.94	26.28	9.72	18.03	23.04	7.68	13.68	17.42	5.73	39.13	42.49	5.16
Goat ..	23.16	30.11	10.48	18.04	23.06	7.56	8.74	11.43	4.05	40.00	45.45	8.21
Rat ..	21.26	24.91	5.95	24.27	27.04	4.53	8.98	11.14	3.52	38.74	43.66	8.03

Note.—S.E. ($\bar{x}$) = S.D./ $\sqrt{n}$

The limits for $\bar{x}$ are $\bar{x} \pm \text{S.E.} (\bar{x}) \times t_{n-1} (P=0.05)$

Upper limit for S.D. is $\frac{\text{S.D.} \times \sqrt{n}}{\sqrt{\text{Chi square}_{n-1} (P=0.975)}}$

TEXT-FIGURE.

*Electrophoretic pattern of serum proteins of goat, cow, rat and man.*

Lower and upper confidence limits for the percentages of proteins in the serum of an individual from the species have also been calculated (vide Table III):—

TABLE III.

Estimated confidence limits for per cent compositions.

(Confidence level = 95 per cent.)

Species.	γ -GLOBULIN.		β -GLOBULIN.		α -GLOBULIN.		ALBUMIN.	
	L.L.	U.L.	L.L.	U.L.	L.L.	U.L.	L.L.	U.L.
Man ..	14.70	32.33	4.18	24.97	6.34	21.14	40.68	56.07
Cow ..	0.88	45.33	2.98	38.09	2.45	28.65	29.01	52.61
Goat ..	2.63	50.65	3.23	37.87	0.80	19.37	23.92	61.54
Rat ..	9.59	36.58	15.40	35.91	2.08	18.05	23.01	59.39

Note.—Lower limit = L.L. ($\bar{x}$) — 1.9600 U.L. (S.D.). Upper limit = U.L. ($\bar{x}$) + 1.9600 U.L. (S.D.).

The confidence limits given in Tables II and III show that the species differ very much in their limits. But there is overlapping of the intervals on a wide scale and it seems that the per cent composition of the proteins is no sure guide for the identification of a given sample. The high variability among goats and cows, which, we feel, can be reduced to some extent if the samples are taken from a more normal population, is another complicating factor.

Relative concentrations.—It is well known that the albumin/globulin ratio in serum is very much altered in diseases. Bal Krishna and Chakravarty (1957) have found in an experiment on rabbits that the A/G ratio is even reversed by induced cholesterol atherosclerosis.

The means, standard deviations, and coefficients of variation for four ratios, viz. albumin/globulin, α -globulin/albumin, β -globulin/albumin and γ -globulin/albumin, are given in Table IV:—

TABLE IV.
Relative concentrations of proteins in the sera of 4 species.

Species.		Albumin	α -Globulin	β -Globulin	γ -Globulin
		Globulin.	Albumin.	Albumin.	Albumin.
Man	{ Mean..	0.940	0.285	0.300	0.487
	{ S.D. ..	0.082	0.047	0.075	0.055
	{ C.V. ..	8.75	16.43	25.12	11.21
Cow	{ Mean..	0.694	0.388	0.501	0.576
	{ S.D. ..	0.094	0.115	0.106	0.182
	{ C.V. ..	13.58	29.67	21.05	31.58
Goat	{ Mean..	0.760	0.239	0.487	0.646
	{ S.D. ..	0.165	0.064	0.125	0.211
	{ C.V. ..	21.77	26.85	25.71	32.63
Rat	{ Mean..	0.715	0.253	0.637	0.578
	{ S.D. ..	0.164	0.087	0.133	0.153
	{ C.V. ..	22.95	34.56	20.84	26.52

Bartlett's test for heterogeneity of the variances showed that it is not permissible to pool the results to get an estimate of the error variance except in the case of the β -globulin/albumin ratio. An analysis of the variance was performed in this case and the results are given in Table V:—

TABLE V.
Analysis of variance for the β -globulin/albumin ratio.

Sources.	Sum of squares.	d.f.	Mean square.	Variance ratio, F.	Remark.
Between species ..	1.17847	3	0.3928	31.43	Significant.
Residual ..	0.87486	70	0.0125		
Total ..	2.05333	73			

The observed value of the variance ratio was found to be 31.431 which gives a *P* less than 0.001, that is, the chance that we will get a value of *F* greater than the observed one is less than one in a thousand. Hence, we infer that the variability between species predominate over the within-species variation. The estimate of the error variance is 0.0125 and hence, the standard error of the difference between the means for a pair of species is $\left(\frac{1}{n_1} + \frac{1}{n_2}\right) 0.0125$, where n_1 and n_2 are the numbers of individuals from the two species. All the differences except that between cow and goat species are highly significant (significant at a level of $P=0.01$).

For all the four ratios, confidence limits were calculated for the means and S.D. (upper limit only) and these are given in Table VI:—

TABLE VI.

Estimated confidence limits for the means and S.D. of Table III.

(Confidence level=95 per cent.)

Species.	ALBUMIN/GLOBULIN.			α -GLOBULIN/ ALBUMIN.			β -GLOBULIN/ALBUMIN.			γ -GLOBULIN/ALBUMIN.		
	Mean.		S.D.	Mean.		S.D.	Mean.		S.D.	Mean.		S.D.
	L.L.	U.L.	U.L.	L.L.	U.L.	U.L.	L.L.	U.L.	U.L.	L.L.	U.L.	U.L.
Man ..	0.902	0.979	0.124	0.263	0.307	0.071	0.265	0.335	0.113	0.462	0.513	0.082
Cow ..	0.646	0.743	0.149	0.329	0.447	0.182	0.447	0.555	0.116	0.483	0.670	0.287
Goat ..	0.672	0.848	0.266	0.205	0.273	0.103	0.421	0.554	0.201	0.534	0.758	0.339
Rat ..	0.640	0.790	0.244	0.213	0.292	0.130	0.577	0.697	0.197	0.508	0.648	0.228

Using these values, upper and lower confidence limits for individual observations of the ratios were also calculated. These are given in Table VII. In all cases the confidence level was taken to be 95 per cent. The formulæ used are the same as those in Tables II and III.

TABLE VII.

Estimated confidence limits for the relative concentrations of the proteins.

(Confidence level=95 per cent.)

Species.	ALBUMIN GLOBULIN.		α -GLOBULIN ALBUMIN.		β -GLOBULIN ALBUMIN.		γ -GLOBULIN ALBUMIN.	
	L.L.	U.L.	L.L.	U.L.	L.L.	U.L.	L.L.	U.L.
	L.L.	U.L.	L.L.	U.L.	L.L.	U.L.	L.L.	U.L.
Man ..	0.659	1.222	0.125	0.445	0.042	0.557	0.300	0.674
Cow ..	0.354	1.034	0	0.803	0.121	0.881	0	1.233
Goat ..	0.151	1.369	0.003	0.476	0.026	0.949	0	1.422
Rat ..	0.162	1.268	0	0.547	0.190	1.084	0.062	1.094

Note.—In the case of negative values for the lower limit zero has been taken as the lower limit. All the zero values were obtained in this manner.

In the case of relative concentrations also, the species are found to differ very much in the level as well as the variability of the ratios. Here again, the results for cows and goats are found to be much variable than those for the other two species. *Homo sapiens* were found to be distinctly higher in the A/G ratio than the other species, rats in the β -globulin/albumin ratio and cows in the α -globulin/albumin ratio (Table VI). For the purpose of identification of an individual sample, relative concentrations have the same drawbacks as per cent compositions.

The confidence limits given in Table VII may be useful in some other ways. If the levels of the ratios observed in the serum of an individual were found to be outside the limits given in Table VII, we may take that individual to be abnormal in some way. Calculation of such limits for persons suffering from diseases known to affect these ratios would be of some help in diagnosis.

Moore and Lynn (1941) have obtained similar ratios for normal human plasma. They have combined their results with those given by Longworth and Scudder and obtained the results given in Table VIII.

TABLE VIII.

Summary of concentration ratios for twenty-five normal plasma patterns.

(Taken from Moore and Lynn.)

	Sample size.	Albumin	α -globulin	β -globulin	Fibrinogen	γ -globulin
		Globulin.	Albumin.	Albumin.	Albumin.	Albumin.
Longworth ..	7	1.99	0.125	0.23	0.09	0.21
Scudder ..	6	2.25	0.10	0.18	0.083	0.17
Moore and Lynn ..	12	1.94	0.126	0.22	0.081	0.19
Average ..	25	1.99	0.12	0.21	0.08	0.19
S.D. ..		0.30	0.025	0.04	0.02	0.04
C.V. (per cent) ..		15	21	19	25	21

In our results, there is no fibrinogen component. The coefficients of variations from our data compare very favourably with those given in Table VIII. Moore and Lynn (*loc. cit.*) have also calculated 1-, 2-, and 3-sigma limits for these ratios. But there are some mistakes in the formulæ they have used, and in some cases the formulæ used by them, do not take into consideration the fact that the sample size is not very large. We have re-calculated the limits for a confidence level of 95 per cent using the correct and appropriate formulæ. The results are given in Table IX.

The re-calculated 95 per cent limits were the same as the 94.5 per cent limits given by Moore and Lynn (*loc. cit.*) up to 2 decimal places (except for one case). These limits are very different from those obtained by us. The A/G ratio from our results is only half that given by Moore and Lynn, whereas the 2 ratios α - $\frac{\text{globulin}}{\text{albumin}}$ and γ - $\frac{\text{globulin}}{\text{albumin}}$ are more than twice those given by them. The β - $\frac{\text{globulin}}{\text{albumin}}$ ratio is not very different in the two series. It would be of very great

They are denoted by (a_{ij}) and (a^{ij}) , respectively.

$$V_{p,k} = \sum_{ij=1}^p a^{ij} \sum_{r=1}^k n_r (\bar{x}_{ir} - \bar{x}_{i.}) (\bar{x}_{jr} - \bar{x}_{j.})$$

where $\bar{x}_{i.} = \sum n_r \bar{x}_{ir} / \sum n_r$ is the mean of the i^{th} variable for all the species together, n_r is the sample size from the r^{th} population, and $\bar{x}_{ir}$ is the mean of the i^{th} variable in the r^{th} population can be used as a chi-square with $p(k-1)$ degrees of freedom to test the hypothesis that the mean values are the same in all the k populations for these variables (Rao, 1945 and 1948).

Here $p=3$ and $k=4$, and $V_{3,4}$ may be treated as a chi-square with 9 degrees of freedom. The observed value of $V_{3,4}$ was found to be 159.2226 and the chance that we will get a value of chi-square greater than this (on 9 degrees of freedom) is less than 0.001. Hence, the mean values are highly significantly different. Thus, the differences in the per cent compositions of the proteins between the species are found to be statistically highly significant.

Once this over-all difference has been established, the next step is to try to get some function of the observations for a pair of species, the value of which would be a guide in identifying individuals from either of these species. We have chosen a linear compound of the observations as the appropriate function (It is the same as Fisher's discriminant function). The functions themselves, mean values of the function for the 2 populations, variances of the functions, standard error of the difference between the two mean values and the value of t are all given in Table XII for the 6 comparisons.

The chance that a higher value for ' t ' than that observed would be obtained is less than 0.001 in all cases. Hence, there is a real difference between any two species.

The means, S.D. (with corresponding degree of freedom) as well as the C.V. for the discriminant functions are given in Table XIII:—

TABLE XIII.

Means, standard deviations and coefficients of variations for values of the discriminant function.

Comparisons.		Mean.	S.D.	d.f.	C.V.
Man and cow	Man ..	55.769	3.504	16	6.28
	Cow ..	65.607	2.838	13	4.33
	Man ..	40.088	5.405	16	13.48
Man and goat	Goat ..	54.989	8.533	12	15.52
	Man ..	49.126	25.111	16	51.12
Man and rat	Rat ..	139.589	24.866	17	17.81
	Cow ..	683.100	113.198	13	16.57
Cow and goat	Goat ..	523.254	68.212	12	13.04
	Cow ..	27.688	12.252	13	44.61
Cow and rat	Rat ..	48.898	8.544	17	17.47
	Goat ..	309.447	64.376	12	20.80
Goat and rat	Rat ..	379.316	46.870	17	12.36

As before, lower and upper confidence limits for the means and the upper confidence limits for the S.D. were calculated. These are given in Table XIV:—

TABLE XIV.

Estimated confidence limits for the means and S.D. of Table XIII.

(Confidence level = 95 per cent)

Comparisons.			MEAN.		S.D.
			LL.	U.L.	U.L.
Man and cow	{ Man ..	54.108	57.430	6.015	
	{ Cow ..	64.120	67.094	5.313	
Man and goat	{ Man ..	37.525	42.651	9.279	
	{ Goat ..	50.340	59.638	16.993	
Man and rat	{ Man ..	37.221	61.031	43.108	
	{ Rat ..	128.141	151.037	41.787	
Cow and goat	{ Cow ..	623.796	742.404	211.895	
	{ Goat ..	486.092	560.416	135.837	
Cow and rat	{ Cow ..	21.217	34.159	23.122	
	{ Rat ..	44.964	52.831	14.358	
Goat and rat	{ Goat ..	274.375	344.519	128.198	
	{ Rat ..	357.737	400.895	78.765	

We find that the limits of the means for the 2 species in each comparison are quite distinct. Thus, the joint consideration of the per cent compositions have yielded results which clearly distinguish between the mean values of the species. The confidence limits for the individual values of the discriminant functions were also calculated and these are given in Table XV:—

TABLE XV.

Estimated confidence limits for values of the discriminant functions.

(Confidence level = 95 per cent.)

Comparisons.			VALUE OF DISCRIMINANT FUNCTION:	
			Lower limit.	Upper limit.
Man and cow	{ Man ..	42.319	69.219	
	{ Cow ..	53.707	77.507	
Man and goat	{ Man ..	19.338	60.838	
	{ Goat ..	17.034	92.944	
Man and rat	{ Man ..	—47.271	145.523	
	{ Rat ..	46.238	232.940	
Cow and goat	{ Cow ..	208.482	1157.718	
	{ Goat ..	219.851	826.657	
Cow and rat	{ Cow ..	—24.102	79.478	
	{ Rat ..	16.822	80.973	
Goat and rat	{ Goat ..	23.107	595.787	
	{ Rat ..	203.358	555.274	

Here, as before, the intervals overlap to a very great extent. To find out with what confidence we could identify a given individual, we considered the extreme case. The means were made to lie on the limits (Table XIV) so that the difference between the means of the 2 species is a minimum. This minimum difference was divided by the sum of the maximum values of the standard deviations and the quotient was used to set up confidence limits for the individual values (using the tables of normal probability integral). The confidence levels for identification in these extreme cases are given in Table XVI:—

TABLE XVI.

Confidence levels for identification.

Comparison.	Confidence, per cent.
Man and cow ..	44·5
Man and goat ..	23·0
Man and rat ..	57·0
Cow and goat ..	14·0
Cow and rat ..	22·7
Goat and rat ..	5·0

The confidence levels vary from 5 per cent in the case of the goat and rat comparison to 57 per cent in the case of the man and rat comparison. Here again, the high variability among cows and goats has revealed itself as a stumbling block in the path of identification.

SUMMARY.

1. The sera from individuals of four species were compared with regard to the per cent compositions and relative concentrations of the proteins.

2. All the available references in the field of comparison between the per cent compositions of species confine themselves to a discussion of the graphic patterns obtained. On the basis of these alone, the conclusion has been drawn that each species possesses a 'characteristic and reproducible electrophoretic pattern'. No attempt has been made to consider the between species variability in relation to the within-species variability. Even though the species differ in both the per cent compositions and the relative concentrations of the proteins, the problem of identification of the individual as belonging to one of a given list of species cannot be tackled with a high level of confidence. A part of the difficulty in this connection is the high variability found among cows and goats, the blood samples for which were obtained from the local slaughter-house. An experiment with a more normal sample would be worthwhile as we have found that even with the very small samples we have taken, the discrimination between human and rat sera is at a moderately high level.

3. As far as we know, the method of discrimination using a linear compound of the per cent compositions of all the proteins has been used here for the first time in connection with electrophoresis.

4. A comparison of our results for the human sera with those given by Moore and Lynn (*loc. cit.*) has revealed a wide divergence between the patterns obtained. It would be of interest to find out the reasons for this divergence. In this connection a study of the variations in the patterns for various levels of malnutrition seems to be desirable.

5. We have given confidence limits for individual values of per cent compositions and relative concentrations of the proteins. The limits for the human sera would be of some assistance in the detection of abnormality in an individual. Calculation of such limits from samples suffering from diseases known to affect the protein patterns would be of great value.

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A PILOT STUDY CONCERNING THE INCIDENCE OF TOXOPLASMOSIS IN THE DELHI AREA AND ITS RELATIONSHIP TO EATING HABITS OF THE POPULATION.

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TOXOPLASMA GONDII is a protozoan which is widely distributed in nature and has been recognized in man as well as in a variety of animal hosts (Eyles and Frenkel, 1952, 1954; Farrel *et al.*, 1952; Frenkel and Friedlander, 1951; Jacobs, 1953, 1956; Jacobs, Melton and Jones, 1952; Kass *et al.*, 1952; Manwell *et al.*, 1945; Nicolle and Conor, 1913; Nicolle and Manceaux, 1909; Olafson and Monlux, 1942; Pinkerton and Weinman, 1940; Sabin, 1939, 1942; Weinman, 1943, 1952; Westphal and Finke, 1950; Wolf, Cowen and Paige, 1939). Infection of human beings with toxoplasma may sometimes produce manifestations of a disease, toxoplasmosis, referred to as the neonatal (Frenkel and Friedlander, *loc. cit.*), infantile (Wolf, Cowen and Paige, *loc. cit.*) or adult form, (Feldman and Sabin, 1949; Frenkel, 1948; Frenkel and Friedlander, *loc. cit.*; Kass *et al.*, *loc. cit.*; Kean and Crocott, 1947; Pinkerton and Weinman, *loc. cit.*; Rawal, Jhala and Patel, 1955; Weinman, 1943, 1952) depending on the period of life during which the organism is acquired; in a large number of instances the time of infection is unknown because of absence of symptoms (Feldman and Sabin, *loc. cit.*; Frenkel and Friedlander, *loc. cit.*; Jacobs, 1953, 1956; Jacobs, Cook and Neumann, 1954; Kean and Crocott, *loc. cit.*; Pinkerton and Weinman, *loc. cit.*).

Although isolation of toxoplasma from the patient or from the animal is the most reliable method of identification of toxoplasmosis, this is not always possible, so that histologic, immunologic, and serologic methods will often have to be substituted. It has to be pointed out that microscopic examination alone is not always sufficient in the differentiation between toxoplasmosis and sarcosporidiosis (Awad and Lainson, 1954) and that there is doubt concerning the specificity of the Feldman-Sabin (Awad, 1954) dye test for toxoplasmosis. However, the toxoplasmin

skin test (Frenkel, 1948) as well as the dye test for toxoplasmosis (Sabin and Feldman, 1948) offer practical means for conducting surveys of the population and are valuable aids in the investigation of cases which are suspected of toxoplasmosis but present no symptoms of activity of this disease (Frenkel, 1948, 1949; Jacobs, Cook and Neumann, *loc. cit.*; Kean and Crocott, *loc. cit.*; Sabin, 1942, 1949 and 1952; Warren and Sabin, 1942).

The frequency of toxoplasmosis in man and animal hosts varies in different parts of the world; the disease has been reported from every continent and all climates (Gard, 1951). The incidence is greater in the older population (Feldman and Sabin, *loc. cit.*; Frenkel, 1948; and Gard, *loc. cit.*) and in warmer climates (Feldman, 1953; Gard, *loc. cit.*).

Although there is evidence of the biologic and immunologic identity of toxoplasma of human or animal origin, the mode of acquisition or transmission from man to man, from animal to man or from animal to animal is yet not known with certainty. On the basis of clinical observations and many animal experiments, it is now generally accepted that neonatal toxoplasmosis is the result of congenital transmission of toxoplasma from the mother to the fetus during pregnancy, particularly if the mother acquires toxoplasmosis during the gestation period (Cowen and Wolf, 1950, 1950*a* and 1950*b*; Eichenwald, 1948; Frenkel and Friedlander, *loc. cit.*; Hellbrugge, 1953; Jacobs, 1956; Wildfuhr, 1954; and Wolf Cowen and Paige, *loc. cit.*). Transmission of toxoplasmosis from animal to animal by oral and peroral routes with infected material has sometimes been successful (Eichenwald, *loc. cit.*; Kozar *et al.*, 1952; Manwell *et al.*, *loc. cit.*) but not in all instances (Frenkel, 1951; Jacobs, Woke and Jones, 1950; Van Thiel, 1949). The possibility of transmission of toxoplasmosis by arthropods has repeatedly been tested (Jacobs, 1953; Nicolle and Conor, *loc. cit.*; Weyer, 1951; Woke *et al.*, 1953) but the results are still equivocal (Jacobs, 1953; Nicolle and *et al.*, *loc. cit.*; Woke *et al.*, *loc. cit.*).

The problem of possible acquisition of toxoplasmosis by ingestion of undercooked contaminated meat as well as by the ingestion of organs of infected animals has received considerable attention (Adams *et al.*, 1949; Cowen and Wolf, 1950; Eichenwald, *loc. cit.*; Farrel *et al.*, *loc. cit.*; Jacobs Woke and Jones, *loc. cit.*; Kozar, Wysocka and Sikorska, *loc. cit.*; Nanwell *et al.*, *loc. cit.*; van Thiel, *loc. cit.*). There is recent re-emphasis on the possibility of toxoplasmosis in human beings due to the consumption of pork that has not been sufficiently heated, similar to the contraction of trichinosis (Weinman and Chandler, 1956); however, on the basis of a serologic survey of elderly, orthodox Jews who denied ever having eaten pork, Jacobs, Cook and Neumann (*loc. cit.*) conclude that there is no convincing evidence that toxoplasma infection in humans is usually acquired by ingestion of pork.

On account of these equivocal data, a pilot study of the prevalence of toxoplasmosis in the Delhi area seemed promising, because of the varied but distinct eating habits of certain groups of the indigenous population, bound by tradition or observed as religious practices. Members of some of these groups do not eat beef but may eat pork, goat or game; others do not eat pork but may eat beef, goat or game many of the people are strict vegetarians. Among the towns people are also many indiscriminate meat eaters,

Goat is the most widely consumed meat eaten by the population in this area; buffalo meat and pork are also commonly eaten while beef is difficult to obtain. Meat of the local wild deer family is greatly appreciated whenever it becomes available. Poultry, particularly chicken, goose, peasant, peacock and partridge is consumed by many, especially among the members of the well-to-do classes.

The information gained from individuals concerning their eating habits with regard to flesh meat is usually correct; however, internal organs, such as liver, brain, lung, spleen or kidney are freely consumed but are sometimes not considered as meat. Therefore, this point has to be clarified in every single case.

MATERIALS AND METHODS.

Skin tests with toxoplasmin were made in all cases and in a few instances the dye test for toxoplasmosis was also employed. 0.1 c.c. of toxoplasmin, diluted with saline 1:1,000 was injected intradermally on one arm and 0.1 c.c. of mouse spleen antigen, diluted with saline 1:1,000 was given on the other arm. The skin reaction to these injections was usually read 24 hours later but some were observed after 48 hours. The extent of erythema was measured and the test was considered positive, when the area reached or exceeded 10 mm. in diameter and had become indurated*.

The age, sex, religion and the detailed eating habits were recorded in every case and in instances where the skin reaction with toxoplasmin was considered positive, the person was given a thorough physical examination for clinical evidence of lesions compatible with toxoplasmosis. Untoward reactions to either the toxoplasmin or the control antigen were not observed. A substantial number of skin reactions could not be read due to lack of co-operation of the individuals being tested. Blood samples were collected from all persons who exhibited a positive skin reaction in order to perform a toxoplasma dye test on the sera; due to inadvertent electric power failure in Delhi, many of the blood samples were spoiled and only three could be carried out.

The toxoplasma skin test was applied to two groups; children between the ages 7 and 15 and adults between the ages 20 and 49 years.

RESULTS.

1. *Children.*—A total number of 198 skin tests and controls was performed of which 168 (85 per cent were read; in 115 instances the results were examined after 24 hours and 53 tests were read after 48 hours. There were 108 boys and 60 girls; their ages varied from 7 to 15 years, giving an average of 10 years of age. Among the children were 57 vegetarians and 111 non-vegetarians of whom 64 had not consumed pork and 47 denied having ever eaten beef.

*Toxoplasmin and control antigen, prepared from the peritoneal exudate of mice infected with toxoplasma was generously supplied by Dr. Leon Jacobs from the Institute of Tropical Medicine, National Institute of Health, Bethesda, MD., U.S.A., and all toxoplasma dye tests were performed in this laboratory. We wish to thank Dr. Jacobs for this kindness as well as for much valuable advice; without this co-operation this study would not have been possible.

Two positive skin reactions with toxoplasmin were obtained in this group: one was a girl, 9 years of age, a vegetarian, who exhibited an area of erythema measuring 25×50 mm. in size and showed some induration 24 hours after injection. The control test remained negative. The toxoplasma-dye test was positive in a titre of 1:256.

The second positive skin reaction occurred in a 12 years old boy, a non-vegetarian, who had consumed various kinds of meat for the last 10 years; he had eaten beef twice or three times a week for the last two years but denied ever having consumed pork.

The percentage of positive skin reactions to toxoplasmin in the children's group was 1.2.

2. *Adults.*—A total number of 140 skin tests and controls was performed of which 120 (86 per cent) were read. There were 58 males and 62 females; 40 were vegetarians and 80 non-vegetarians of whom 44 denied having ever eaten beef and 35 ever having eaten pork. One was an indiscriminate meat eater. The total number of vegetarians in this group was 40 and that of non-vegetarians 80. The ages were distributed as follows:—

20 to 29 years of age	...	42	1 positive skin test.
30 to 39 years of age	...	57	3 positive skin tests.
40 to 49 years of age	...	21	2 positive skin tests.

The toxoplasmin-skin test was positive in five instances; four were males, 28, 30, 32, and 40 years of age, respectively, one was a female 45 years of age. All five were non-vegetarians and three of them had eaten various kinds of meat such as mutton, pork, beef, chicken as well as organs of animals, such as liver, brain, kidney, lung and spleen but no beef. Two members of this group denied ever having eaten pork or the organs of swine, but had eaten beef.

One toxoplasmin-skin test performed on a 35-year old female vegetarian, who had been admitted to the Irwin Hospital, New Delhi, with the diagnosis of chorioretinitis, gave a doubtful positive reaction showing an area of erythema measuring less than 5 mm. in diameter and exhibiting some induration. The test was repeated within 3 days with similar results. The control test remained negative. The toxoplasma-dye test was positive in a titre of 1:256. In view of the outcome of this test and the presence of chorioretinitis, the case was considered one of toxoplasmosis despite the doubtful positive skin test.

The overall percentage of positive skin reactions to toxoplasmin in the adult group was 5.

DISCUSSION.

The outcome of this pilot study indicates that toxoplasmosis does occur in the Delhi area and that the incidence of this condition is about the same as that reported by Rawal, Jhala and Patel (*loc. cit.*) from Bombay. This survey also indicates that presumptive evidence of toxoplasmosis is more frequently demonstrated in adults than in children.

The preponderance of positive outcomes of tests for toxoplasmosis in the group of non-vegetarian adults suggests the possibility of acquisition of this

condition by eating insufficiently heated infected meat or organs of infected animals. The significance of this important point has to be probed in a study of a larger group of cases.

SUMMARY.

1. Skin tests for toxoplasmosis of a group of 168 children between the ages of 7 and 15 years gave positive results in 2 instances, a 9 year old girl, a vegetarian and a 12 year old non-vegetarian boy who had never eaten pork. Fifty seven children were vegetarians and 111 non-vegetarians. The overall percentage of positive skin tests in children was 1.2.

2. Skin tests for toxoplasmosis performed on 120 adults between the ages of 20 and 49 years gave positive results in 5 instances; two males, 28 and 48 years of age, respectively, and a woman 45 years of age, all non-vegetarians who denied ever having eaten beef and two were males, 32 and 33 years of age respectively who had never eaten pork.

One doubtful skin reaction was obtained in a woman 35-year old, a vegetarian, who had been admitted to the hospital with diagnosis of chorioretinitis. The toxoplasma-dye test of the serum of this patient was positive in a dilution of 1:256. Despite the weak skin reaction, this case was classified as one of toxoplasmosis. The overall number of positive skin tests with histoplasmin was, therefore, 6; five persons were non-vegetarians and one was a vegetarian.

3. With the exception of the case of chorioretinitis, none of the other persons exhibiting a positive-skin test for toxoplasmosis showed any clinical evidence of this condition on physical examination; for this reason they were classified as latent instances of toxoplasmosis.

4. Among the total number of 191 non-vegetarians in both groups, five exhibited positive skin reactions, while 2 positive reactions occurred among 97 vegetarians. In the adult group alone, however, there were 5 instances of positive skin reactions among 80 non-vegetarians against one positive skin reaction among 40 vegetarians. In view of the comparatively small number of cases examined, the result of this pilot study should be tested in a larger survey.

7. The type of meat eaten does not seem to play any role in the occurrence of toxoplasmosis.

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EFFECT OF TESTOSTERONE PROPIONATE ON THE THYROID OF THIOUREA-TREATED RATS.

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It has been reported in different species of animals that testosterone stimulates the thyroid gland (Nathanson *et al.*, 1940; Money, 1950; Money *et al.*, 1950; Burris *et al.*, 1953; Roy *et al.*, 1956). This stimulation is presumably brought about by an enhancement of TTH secretion of the pituitary (Roy *et al.*, 1956) and the androgen seems to have no direct stimulating action on the thyroid (Roy *et al.*, 1956; Zalesky *et al.*, 1941).

Testosterone has also been claimed to exert a beneficial influence on the thyroid of goitrogen-treated animals. Voitkevich (1950) reported that methyl testosterone caused a considerable decrease of hypertrophy of the thyroid and prevented the complete loss of biological activity of the gland of thiouracil-treated guinea-pigs and cocks. Kar and Sur (1953) also noted that the typical hyperplastic changes of the acinar cells of the thyroid of thiourea-treated rats was reduced following simultaneous treatment with testosterone propionate. On the other hand, Leathem (1947) observed that testosterone propionate had no effect on the thyroid hypertrophy of thiourea-treated rats. Segaloff (1944) and Leathem (1948) failed to note any change in the weight of the thyroid of thiourea-treated rats after androgen administration. It is, thus, seen that the action of testosterone on the thyroid of goitrogen-treated animals is a controversial issue. The true nature and mechanism of influence of the testoid on such goitrogen-treated thyroid is not clear and awaits proper elucidation. As the conditions under which testosterone had to act are expected to be different in the normal and thiourea-treated animals, it was thought worthwhile to re-investigate the above problem in a more detailed manner.

Thiourea was administered to rats for varying periods with the idea of producing different degrees of thyroid insufficiency. Such rats were treated with testosterone propionate and the response of the thyroid was studied.

EXPERIMENTAL PROCEDURE.

The present study comprises three separate experiments. Male albino rats of the Institute colony were used in these investigations.

Experiment No. I.—Twenty-four rats weighing 59.7 ± 2.8 g. were divided into three groups (A, B and C) of 8 each. To group B thiourea (in 0.4 c.c. distilled water) was administered through subcutaneous injections for 21 days. The dose of thiourea was 4 mg./rat daily to start with; it was gradually raised to 10 mg. within 5 days and the maximum dose was maintained for the rest of the experimental period. Group C, in addition to thiourea in the above dose schedule, received simultaneous treatment with testosterone propionate (4 mg. in 0.2 c.c. sterile sesame oil/rat on alternate days) through intramuscular route. The remaining group served as a control and only the diluents were injected to them. All of them were kept under uniform husbandry conditions.

The animals were sacrificed on the day following the last treatment. The thyroid glands were dissected out, weighed and fixed in 10 per cent neutral formalin. Serial paraffin sections of the glands were stained with Ehrlich's hæmatoxylin followed by alcoholic eosin.

Experiment No. II.—Twenty-seven rats weighing 52.0 ± 5.9 g. were employed in this experiment. The animals were likewise divided into three groups (A, B and C) of 9 each. To groups B and C thiourea was administered daily for 38 days. The dose of thiourea was gradually raised from 4 mg. to 8 mg. within a period of 14 days. Subsequently, the maximum dose was maintained for the rest of the experimental period. The animals of group C received, in addition, injections of testosterone propionate (6 mg. in 0.2 c.c. of sterile sesame oil/rat on alternate days) from the 19th day of thiourea treatment. Ten such injections were given over the period of 19 days. Group A served as the control receiving only the diluents.

The rest of the experimental procedures were the same as the previous ones.

Experiment No. III.—Twenty-four rats weighing 40.0 ± 4 g. were divided into three groups of 8 each. Groups B and C received subcutaneous injections of thiourea (in 0.5 c.c. of distilled water) for 88 days. The dose of the drug was gradually increased from 4 mg. to 20 mg. within the period of first 20 days and the maximum dose was maintained for the rest of the experimental period. The animals of group C received, in addition, injections of testosterone propionate (8 mg. in 0.25 c.c. sterile sesame oil) on alternate days. The androgen treatment was spread over 23 days. Group A served as a control and received the injections of the diluents.

The remaining procedures were the same as those of the other two experiments.

RESULTS.

The data on the thyroid weight of the three experiments are presented in Table:—

TABLE.

The thyroid weight and body-weight of the control, thiourea-treated and thiourea-plus testosterone propionate-treated animals.

Group and treatment.	MEAN BODY-WEIGHT (G.),		Mean thyroid weight (mg.) with S.E.
	Initial.	Final.	
<i>Experiment 1.</i>			
A. Control	60.2	86.5	4.7±0.27
B. Thiourea	60.2	79.5	7.8±0.42
C. Thiourea plus testosterone propionate	59.1	73.5	8.8±0.36
<i>Experiment 2.</i>			
A. Control	49.7	97.9	4.9±0.33
B. Thiourea	50.2	89.0	8.8±0.30
C. Thiourea plus testosterone propionate	56.8	98.0	6.5±0.24
<i>Experiment 3.</i>			
A. Control	40.1	147.7	5.0±0.28
B. Thiourea	40.0	125.5	9.3±1.09
C. Thiourea plus testosterone propionate	39.9	120.8	3.8±0.13

ANALYSIS
(Thyroid weight).

Experiment 1.

Group A vs. group B— t 6.20; $P < .001$ (Significant)
 Group A vs. group C— t 9.10; $P < .001$ (Significant)
 Group B vs. group C— t 1.81; $P < .1$ & $> .05$ (Insignificant)

Experiment 2.

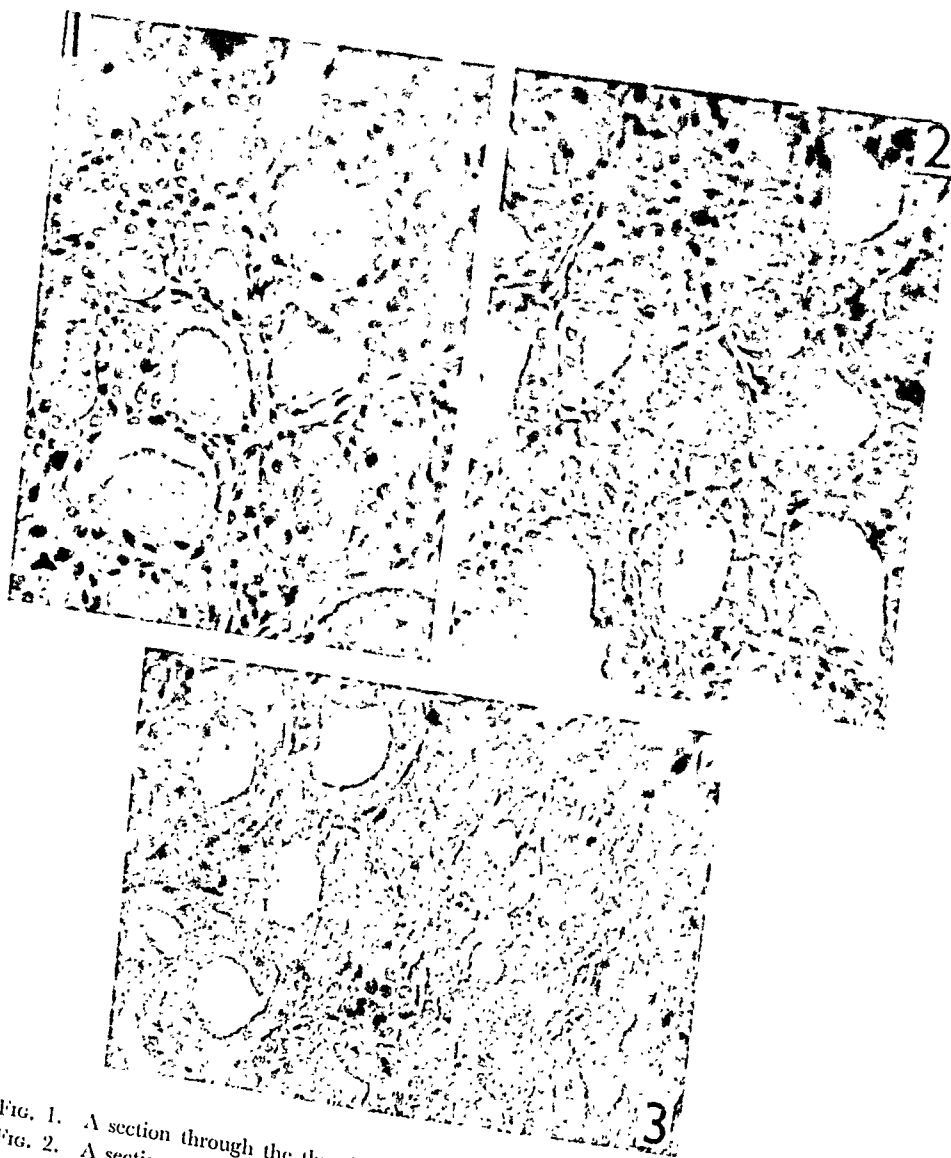
Group A vs. group B— t 8.86; $P < .001$ (Significant)
 Group A vs. group C— t 3.92; $P < .01$ and $> .001$ (Significant)
 Group B vs. group C— t 6.05; $P < .001$ (Significant)

Experiment 3.

Group A vs. group B— t 3.83; $P < .01$ and $> .001$ (Significant)
 Group A vs. group C— t 3.89; $P < .01$ and $> .001$ (Significant)
 Group B vs. group C— t 5.04; $P < .001$ (Significant)

Experiment I.—It will be seen that the weight of the thyroid is increased following thiourea administration. The weight of the gland in the combined treated group does not show any appreciable difference from that of the goitrogen-treated ones.

Histological picture of the thyroid of the animals in the control group present a normal appearance (Plate XVII, fig. 1). The follicles are filled with acidophilic colloid which shows a few vacuoles. The cells lining the acini are of



- FIG. 1. A section through the thyroid of a normal rat.
 FIG. 2. A section through the thyroid of thiourea-treated rat of 'Experiment I'. Note the hyperplastic changes in the acinar cells and the change in acinar colloid content. Compare with Fig. 1.
 FIG. 3. A section through the thyroid of thiourea *plus* testosterone propionate treated rat of 'Experiment II'. Note the hyperplastic changes in the acinar cells and the change in acinar colloid content. Compare with Figs. 1 and 2.

testosterone can influence the activity of such thyroid can be considered along the following lines. Testosterone has been shown to stimulate the normal thyroid gland and this is due to augmentation of TTH output of the pituitary (Roy *et al.*, 1956). That such a stimulation of the thyroid gland is associated with an increase in the thyroid hormone secretion becomes relevant from the fact that testosterone elevates BMR (Thompson, 1941; Kenyon, 1942). Due to this ability of the testoid to enhance the elaboration of TTH from the pituitary, there will be a tendency for it to increase the overall TTH secretion in the thiourea-treated animal. Thyrotrophic hormone, in its turn, has been shown (Bastenie and Kowaleski, 1950) to increase the oxygen consumption of the thyroid in the thiourea-treated animals, though to a lesser extent than it can do in normal ones. Moreover, it has also been demonstrated that TTH increases the peroxidase activity of the thyroid (Villamil and Mancini, 1947). From these observations it seems possible that testosterone might improve the secretory activity of the thyroid of goitrogen-treated animals by increasing the TTH secretion from the pituitary. In that case, an enhanced secretion of thyroid hormone will tend to inhibit the output of TTH, which was previously geared up as a result of lack of thyroid hormone induced by thiourea administration.

It is not known whether testosterone can influence directly the peroxidase activity of the thyroid gland. If it possessed any such property it could be expected to counteract the action of thiourea and prevent any hyperplastic changes caused by the latter.

It is pertinent to note here that testosterone has been reported to stimulate the adrenal cortex (Rennels *et al.*, 1953; Castellani, 1952; Roy *et al.*, 1957). The adrenal cortical hormone has been shown to inhibit the thyroid gland producing depression of the acinar epithelium with corresponding rise in the acinar colloid content (Marthi and Rasanen, 1954). Such depressing action of corticoid on the thyroid activity has been reported by other investigators as well (Antopol, 1950; Money *et al.*, *loc. cit.*; Hill *et al.*, 1950; Wolfson *et al.*, 1950; Fredrickson *et al.*, 1952). It may, therefore, be commented that any increased adrenocortical activity induced by testosterone is likely to influence the thyroid.

From the present data, it is seen that the administration of thiourea invariably causes an increase in the weight of the thyroid and produces hypertrophy and hyperplasia of the acinar cells. The changes induced by testosterone propionate are, however, different in the three experiments. In experiment I, there is no significant difference in the weight of the thyroids in thiourea treated and combined treated animals. From the histological study of the thyroids it appears that following simultaneous treatment with testosterone the hyperplastic changes in the thyroid are somewhat increased. This tendency of increasing the thyroid hyperplasia can be explained by the ability of testosterone to augment the TTH secretion from the pituitary. The absence of any significant change in the weight or histology of the thyroid is probably due to the simultaneous suppressing actions of the increasingly secreted thyroid hormone and of the hypertrophied adrenal gland*, which was caused by the testoid influence.

*Mean adrenal weight (mg.) with S. E.

Group A— 19.7 ± 0.56

Group B— 16.0 ± 0.76

Group C— 22.1 ± 1.07

In experiment II, the weight of the thyroid is diminished and the hyperplastic changes in the thyroid are absent following treatment with testosterone and the thyroid presents more or less normal picture. This change in the thyroid is difficult to explain. The improved hormonal secretion of the thyroid of goitrogen-treated animals under the androgen influence may depress the hypophyseal TTH secretion and thus tend to diminish the hyperplastic changes in the thyroid. However, it is to be considered whether it would be possible for the goitrogen-treated thyroid to secrete such an amount of thyroid hormone in the absence of any hyperplastic change, in case the androgen has no direct stimulating effect on the peroxidase activity of the gland. From the results of the third experiment it appears that testosterone has no such direct effect (*vide infra*). It is noteworthy, however, that the adrenal glands of the hormone treated animals were very much hypertrophied*. Probably, the increased secretion of the corticoid hormone from the hypertrophied adrenals plays an important part in depressing the thyroid hyperplasia. Such change was not observed in the first experiment, probably because the dose of testosterone was low and adrenal enlargement was not marked in that case.

In experiment III, the weight of the thyroid in the hormone-treated animals is very much diminished and the histological examination reveals marked atrophy of the acinar cells. In this experiment where the goitrogen was administered in higher doses and for longer period, the thyroid showed very marked hyperplastic changes. Further stimulation by testosterone probably leads to exhaustion with eventual atrophy of the gland. Of course, the possibility of exhaustion at the pituitary level cannot be ruled out. Had the testoid any direct beneficial effect on the peroxidase activity of the thyroid, the gland should not have atrophied.

Any probable suppressing influence of the cortical hormone on the thyroid cannot be thought of in this case as the adrenals were not hypertrophied in comparison to those of the controls†.

SUMMARY.

Administration of thiourea causes an increase in the weight of the thyroid gland and produces hypertrophy and hyperplasia of the acinar cells, the degree of hyperplasia varying roughly with the duration of treatment. Testosterone administration produces varied changes in such thyroids. Various factors, such as duration of thiourea administration, stage of thyroid hyperplasia at which hormone therapy is instituted, the dose of testosterone and the state of adrenocortical activity as influenced by the testoid, probably determine the ultimate response of the thyroid of goitrogen treated rats to testosterone treatment.

The authors want to thank Dr. B. Mukerji for the keen interest he has shown in this work.

*Mean adrenal weight (mg.) with S. E.

Group A— 19.7 ± 0.49
Group B— 16.3 ± 0.47
Group C— 28.0 ± 0.91

†Mean adrenal weight (mg.) with S. E.

Group A— 22.7 ± 1.06
Group B— 17.5 ± 0.75
Group C— 23.4 ± 1.21

Langerhans in animals. Similar observation, on the islands of Langerhans in animals as a result of administration of thyroid in small doses, was made by Haist (1953) and Kinash and Haist (1955). Formation of new islets in the pancreas of animals after administration of proper dose of desiccated thyroid was observed by Houssay, Foglia and Martinez (1946). Increase in the insulin content of the pancreas of animals after administration of thyroxin in proper doses was observed by Zunz and LaBarre (1932) and Fraenkel Conrat *et al.* (1942).

Anterior pituitary extract (A.P.E.) when administered in suitable doses produce proliferation of β cells of islands of Langerhans in animals. This was noted by Richardson and Young (1938). The above finding was confirmed by Ham and Haist (1939, 1941) who observed that under similar conditions, acinar cells of the duct also proliferated giving rise to the formation of new β cells.

Marks and Young (1940), Soong (1940), Fraenkel Conrat *et al.* (1941) and Funk *et al.* (1941) observed earlier that injection of pituitary extract to rats caused increase in the insulin content of their pancreas.

Though insulin causes universal lowering of blood sugar in all types of diabetes, yet long continued administration of insulin may, as pointed out by Haist *et al.* (1949), arrest the growth of islet tissues. On the other hand, chronic and continued hyperglycemia may cause progressive deterioration of the pancreatic lesion in experimental diabetes (Luken's hyperglycemic theory). Thus, it is reasonable to assume that proper dose of insulin, if combined with agents which will stimulate the pancreatic β cells, may produce better effect towards the regression of diabetes than either of them alone. This view is supported by the observations of Rodriguez (*loc. cit.*) who obtained better effect by combining oestrogen with insulin in alloxan diabetic rats than with oestrogen alone. Oestrogen in combination with glucose, was also observed by Mukherjee *et al.* (1956) to produce better effect in alloxan diabetic rats than oestrogen alone.

In view of the above findings, the study of the effect of administration of corticotrophin, desiccated thyroid and insulin separately and in combination of the former two with insulin on the incidence and course of alloxan diabetes has been undertaken.

MATERIALS AND METHODS.

Two hundred and forty albino rats of C.D.R.I. colony, of both sex, were used in this experiment. Animals were housed in separate cages and fed on identical stock diet for a period of one week before starting of experiment.

One group of 140 animals was treated as follows:—

1. Regular insulin 1 unit/rat/day was injected subcutaneously for a period of 7 days to 6 rats and for a period of 14 days to other 6 rats.
2. Regular insulin 2 unit/rat/day was injected subcutaneously to 6 rats for 7 days and to another 6 rats for 14 days.
3. Desiccated thyroid suspended in water 100 mg./rat/day was forced fed to 13 rats for 6 days and to another 30 rats for 12 days.
4. Corticotrophin (Organon) 2 mg./rat/day was injected intramuscularly to 6 rats for 7 days and to 10 rats for 14 days.

On completion of treatment at different periods as mentioned above, the animals were kept without food for 24 hours (water allowed *ad lib.*). Alloxan in 6 per cent solution was then injected subcutaneously in the dose 175 mg./kg. to all the above 83 treated animals as well as to another 57 untreated normal animals under similar fasting condition. The latter served as untreated control. Animals suffering from glycosuria and ketonurea were treated with 4 units of regular insulin twice daily to enable them to survive. Insulin injection was preceded by forced feeding of milk. Blood sugar was estimated twice during first week on the 2nd and 7th day after alloxan administration.

The next group of 100 animals was given subcutaneous injection of alloxan in the same dose and under similar fasting condition as stated above. The animals suffering from glycosuria and ketonurea were also treated with insulin and forced fed with milk as stated before. The surviving animals were then treated as follows:—

1. Subcutaneous injection of regular insulin 4 units/rat/day was continued in 9 animals which served as control.
2. Desiccated thyroid 10 mg./rat/day was fed to 8 animals (but no insulin).
3. Intramuscular injection of corticotrophin 0.1 mg./rat/day was given to 9 rats (but no insulin).
4. Desiccated thyroid was fed and insulin injected to 16 animals in the same dose and same way as in (1) and (2).
5. Corticotrophin and insulin was administered to 9 animals exactly in the same dose and same way as in (1) and (3). Treatment was continued for 8 weeks.

Blood sugar was determined and weight recorded of all the animals of both groups once every week during the period of observation of 8 weeks. Treatment was discontinued in animals whose blood sugar had gone below 0.200 per cent anytime during the course of treatment and the animals were followed up. The treatment was, however, re-started in animals whose blood sugar had again gone beyond 0.200 per cent. Pancreas were collected of all the animals at the end of 8th week and also of the animals who died during observation. Blood for sugar determination was collected in all instances in 24 hours fasting condition. Blood sugar was estimated by Folin and Wu (1920) method, sections of the pancreas were stained by Gomori's chromealum hæmatoxyline-phloxine and hæmatoxylin-eosin methods.

RESULTS.

Effect of pre-treatment with regular insulin, desiccated thyroid and corticotrophin on the diabetogenic action of alloxan and on the course of alloxan induced diabetes in albino rats has been given in Table I.

The results show that pre-treatment with regular insulin in small (1 unit) or large (2 units) doses for short (7 days) and long (14 days) periods do not either protect the albino rats from the diabetogenic action of alloxan or modify the course

EXPLANATION OF PLATES XIX AND XX.

Section of the island of Langerhan's of a diabetic rat treated by insulin (S.C.) and thyroid (oral) $\times 420$.

The islands of Langerhan's in the combined insulin-thyroid treated group of rats which recovered shows the following features. Number of β cells are less than in normal. Cell outlines although not absolutely clear is still discernible. The nuclei are spherical and arranged in the centre. The cytoplasm is more granular than in any of the two other mentioned groups (Fig. 1).

Section of the island of Langerhan's of a diabetic rat treated by insulin only. The island of Langerhan's in the insulin-treated diabetic rats bear a typical appearance of chronic diabetes. There are few β cells only and cell outline of the cells cannot be seen. The nuclei are shrunken and vacuolated. The cytoplasm is absolutely agranular (Fig. 2).

Section of the island of Langerhan's of a diabetic rat treated by thyroid only.

The island of Langerhan's in the thyroid treated group represents more advanced degree of degeneration as follows. The β cells are completely necrosed and are disintegrated. Only the nuclear remnants can be seen. No cytoplasmic granules are seen, only amorphous remnants are found (Fig. 3).

Section of the island of Langerhan's of a diabetic rat treated by corticotrophin only.

The islands of Langerhan's of corticotrophin-treated group of rats bear evidence of degeneration as follows:—

1. Number of β cells are less and no cell outline can be seen in many cells.
2. The nuclei are not spherical in many cases, are irregularly scattered. Some degenerated nuclei can also be seen.
3. The cytoplasm is almost agranular except in a few peripheral cells (Fig. 4).

Section of the island of Langerhan's of a diabetic rat treated by corticotrophin and insulin.

The island of Langerhan's of corticotrophin and insulin-treated group of rats bear the degenerative nature of the β cells is evident from the following:—

1. Although a fair number of β cells are present except in the central zone, the nuclei in most of them are strophic and are irregularly placed in many instances.
2. No definite cell outline is recognized yet the cytoplasm is more granular than in corticotrophin or insulin-treated group (Fig. 5).

Section of the island of Langerhan's of a (untreated) diabetic control rat (Fig. 6).

Section of the island of Langerhan's of a normal rat (Fig. 7).

Section of the island of Langerhan's of a diabetic rat treated by thyroid and insulin.

A highly magnified section of the pancreas from a diabetic rat treated with thyroid and insulin, showing (i) loss of demarcation of the acinar epithelium and β cells of the islands of Langerhan's and (ii) extrusion of the nuclei and granules from some acinar cells (Fig. 8).

(N.B.—Figures 6 and 7 are sections of normal and diabetic controls for comparison only).

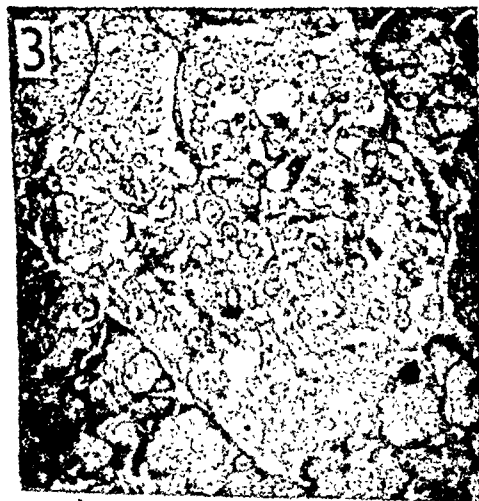
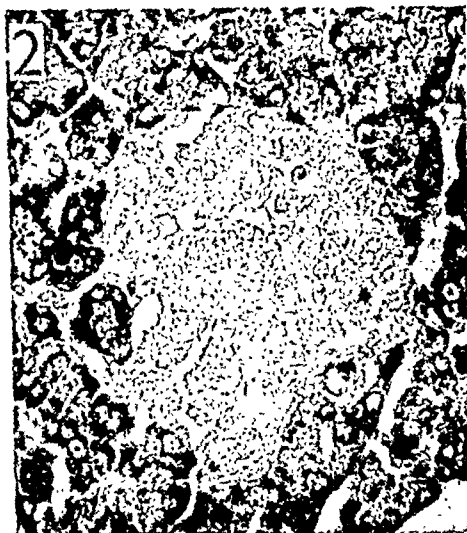
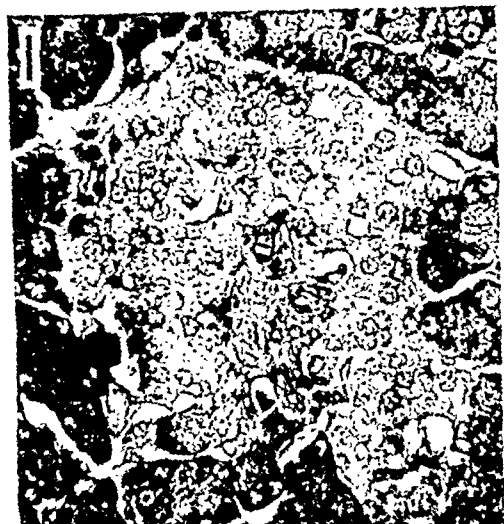


TABLE II.

Effect of insulin, desiccated thyroid and corticotrophin on course of alloxan induced diabetes in albino rats.

Number of diabetic rats used.	Dose of drug used.	Duration of treatment, week.	Number of rats recovered.	Per cent, recovery.	Diabetes, per cent.
9	Regular insulin 4 μ /rat/s.c./daily	8	0	0	100
8	Desiccated thyroid 10 mg./rat/oral/daily	8	1	12	88
9	Corticotrophin 0.1 mg./rat/i.m./daily	8	0	0	100
16	Insulin 4 μ /rat/s.c./daily plus desiccated thyroid 10 mg./rat/oral/daily	8	8	50	50
9	Insulin 4 μ /rat/s.c./daily plus corticotrophin 1 mg./rat/i.m./daily	8	0	0	100

Recovery is indicated by normoglycemia and arrest of loss of weight or gain in weight above the pre-alloxan level.

Results showed that alloxan-induced diabetic condition in rats could not be modified in any way when the animals are treated either with insulin and corticotrophin separately or in combination. When the alloxan-induced diabetic rats were treated with desiccated thyroid (10 mg. daily) alone (1 out of 8) 12 per cent of the animals recovered from diabetes and when the combined treatment of desiccated thyroid and insulin was given, (8 out of 16), i.e., 50 per cent of the animals recovered from diabetic condition.

DISCUSSION.

The results show that pre-medication of rats with insulin or corticotrophin cannot modify the incidence of diabetes after alloxan administration. The results further show that corticotrophin alone or in combination with insulin-produce no ameliorative effect whatsoever in alloxan-induced diabetic condition. Though insulin is beneficial in controlling hyperglycemia, it possesses no pancreatropic action. Haist *et al.* (*loc. cit.*) demonstrated that long continued administration of insulin caused atrophy of the islands of Langerhans. Majority of workers agree that prolonged use of insulin suppresses the metabolic activity of the β -cells and causes atrophy of the islands of Langerhans. Best *et al.* (1955), in a review article, indicated that majority of opinion was in agreement that prolonged use of insulin would suppress formation and secretion of insulin and the islands would undergo disuse-atrophy to such an extent that finally diabetes would result (Haist, *loc. cit.*; Bell, 1953). The results obtained by us that insulin treatment has no effect on the incidence and cause of alloxan diabetes in albino rats and that insulin treatment increases the sensitivity of the albino rats to the diabetogenic action of alloxan are supported by the above findings.

Jensen and Grattan (1950) observed that corticotrophin definitely antagonizes the effect of insulin and prevent insulin hypoglycemia. Through the action of

cortico-adrenal hormone ACTH antagonizes insulin action on the hexokinase reaction and retards glycolysis of glucose, ACTH also causes increased peripheral demand of insulin due to increased rate of protein (and fat) catabolism. All these result in a vicious circle which increase the glycemia level and thereby cause damage to the pancreatic β cells. Abraham *et al.* (1953) observed that prolonged injection of corticotrophin increases the size of islands of Langerhans and causes enlargement and degranulation of β cells suggestive of exhaustion and atrophy of the insular apparatus. Cavallero and Mosca (1953), at the same time, found that corticotrophin injection stimulates chiefly the alpha cells as is evidenced by their increased mitotic activity. All the above findings go to show that corticotrophin injection creates a situation favourable for production of diabetes rather than either to prevent the diabetogenic action of alloxan or to ameliorate the diabetic conditions produced by alloxan. Therefore, the failure of corticotrophin to prevent diabetogenic action of alloxan and to exert any beneficial effect either by corticotrophin alone or in combination with insulin on the alloxan induced diabetic condition as observed by us can be explained on the above light. Histological pictures of the islands of Langerhans showing necrosed character of the β -cells (Plate XIX, figs. 1, 2, 3 and 4) confirm the findings.

Results of pre-administration of desiccated thyroid show that when thyroid is fed for a short time (6 days), it protects (6 out of 13) 46 per cent of the animals from the diabetogenic action of alloxan but no such protective action is exhibited when pre-feeding is continued for a longer period (12 days). The results further show that when alloxan-induced diabetic rats were treated with thyroid alone, one out of eight animals (12 per cent) recovered from diabetes as is evidenced by normal blood sugar, absence of glycosuria and gain in weight (re-gain of lost weight). But the histological picture of the pancreas of this animal still shows necrosed condition of the β cells. Better effect was obtained by us when thyroid feeding was combined with insulin treatment. Out of 16 animals which received combined treatment 8 (50 per cent) recovered from diabetes as judged by their normal blood sugar level, absence of glycosuria and re-gain of the lost weight.

Although the tissue response is not complete, evidence of formation of new β cells from acinar epithelium can be suggested from the following findings:—

- (i) Loss of demarcation between acinar cells and β cells.
- (ii) Extrusion of the acinar cell nucleus and granules in the β cell region can be seen and although no mitotic figure can be seen in the β cell nucleus in some instances a few normal β cells can be noticed.

Houssay (1946) demonstrated that thyroid feeding to alloxan-induced diabetic rats, caused early and spontaneous regression of their diabetic conditions. This he ascribed to the pancreatropic effect of thyroid hormone. Houssay, Foglia and Martinez (*loc. cit.*) observed that desiccated thyroid administration caused formation of new islets in the pancreas. So, the results of thyroid administration obtained by us are supported by above observations.

In conclusion, it can be mentioned that rats may completely recover from alloxan diabetes when thyroid feeding is simultaneously combined with insulin therapy. The mechanism of such improvement is due to the pancreatropic effect of thyroid on the β cells which is greatly assisted by the hypoglycemic action of

insulin, which prevents further damage of β cells by persistent hyperglycemia of diabetes.

SUMMARY.

1. Effect of pre-treatment with insulin, corticotrophin and desiccated thyroid separately on the diabetogenic action of alloxan and on the course of alloxan induced diabetics as well as the effect of combination of corticotrophin with insulin on desiccated thyroid with insulin on the course of alloxan diabetes in albino rats has been studied.

2. Pre-treatment with insulin or corticotrophin has no effect on the diabetogenic action of alloxan or on the course of alloxan induced diabetics in albino rats. Pre-treatment with insulin appears to increase the sensitivity of the rats to diabetogenic action of alloxan.

3. Insulin or corticotrophin or their combination do not ameliorate the alloxan induced diabetic condition in any way.

4. Desiccated thyroid feeding for short time shows some protective effect against the diabetogenic action of alloxan. Treatment with desiccated thyroid alone causes recovery of one out of 8 animals from alloxan-induced diabetics. The combined insulin and thyroid treatment, however, shows better effect and 50 per cent of the animals recovered from alloxan-induced diabetes.

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Ind. Jour. Med. Res., **46**, 3, May, 1958.

EXPERIMENTAL PEPTIC ULCERATION.

Part III.

EFFECT OF ANTICHOLINERGIC DRUGS ON HISTAMINE-INDUCED ULCERATION.

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GRIMSON, Lyons and Reeves (1950) suggested that anticholinergic drugs had a definite place in the treatment of peptic ulceration and were potent tools in the control of ulcer pain and inhibition of the volume of gastric juice. It was

also suggested by them that the need for surgery might be lessened as these drugs would perform so-called 'medical vagotomy'. Ruffin *et al.* (1953) later observed that there still existed a considerable difference of opinion as to the exact value of these drugs in peptic-ulcer therapy and, therefore, a realistic assessment may give a valid appraisal. Lichstein *et al.* (1956) pointed out that further studies on specific physiological mechanisms in the production of gastric secretion in relation to anticholinergic drugs may help to find out a useful therapeutic agent in the treatment of peptic ulcer. Information regarding the specific mode of action of these drugs on histamine-induced ulceration is not clear, although histamine forms part of the normal gastric secretory mechanism and is present in gastric juice (Code, 1956) and all parts of gastric mucosa (Gregory, 1956). In the present communication, therefore, the action of anticholinergic drugs on histamine-induced ulceration has been studied and relative effectiveness on anti-gastric ulcer activity observed.

The effect of anticholinergic drugs on gastric secretion has been studied both in experimental animals and in patients with gastric and duodenal ulcers (Longino *et al.*, 1950; Rider *et al.*, 1953; Atkinson, 1954; Kirsner, Levin and Palmer, 1954; and Roth *et al.*, 1956), but effect of these drugs on peptic activity, however, remains uncertain. Singh, Zaidi and Balkrishna (1958) showed a positive relationship between increased peptic activity and ulceration. The action of anticholinergic drugs on peptic activity in relation to histamine-induced ulceration has, therefore, been investigated. Zaidi and Mukerji (1958) have pointed out the significance of mucous barrier in experimental gastric ulceration and an investigation on the effect of anticholinergic drugs on gastric mucous secretion has also been conducted.

MATERIAL AND METHOD.

Animals.—Male guinea-pigs of average weight, 500 g. were used.

Drugs.—The following anticholinergic compounds were investigated:—

1. *Atropine sulphate*.
2. *Oxyphenonium bromide* is a synthetic anticholinergic quaternary ammonium compound. It is chemically diethyl-(2-hydroxyethyl)-methylammonium bromide- α -phenylcyclo-hexaneglycolate.
3. *Scopolamine methylbromide* is the N-methyl-bromide (quaternary ammonium derivative) of scopolamine.
4. *Pro-banthine* is a synthetic compound, β -di-isopropylaminoethyl xanthene-9-carboxylate methylbromide which is a quaternary amine.
5. *Esentil* is a synthetic new preparation which is 6-beta-diethylamino-ethyl-1-cyclo-hexylcyclohexancarboxylate hydrochloride.

Dose.—Oxyphenonium bromide, atropine sulphate, scopolamine methylbromide, pro-banthine and esentil were dissolved in distilled water (0.5 mg./c.c.) and used in a single dose of one c.c.

Production of peptic ulcer.—Acute peptic ulceration was produced with massive doses of histamine (50 mg.) in guinea-pigs protected with an anti-histaminic, promethazine hydrochloride (Zaidi and Mukerji, *loc. cit.*).



FIG. 1. Superficial ulceration. Complete destruction of mucosa. H and E $\times 30$.



FIG. 2. Deep ulceration. Only thin serous layer intact. H and E $\times 30$.

Experimental procedure.—The animals were divided into six groups and in each fasting contents were aspirated as far as possible. Group I consisted of fifteen animals, group II and V of fourteen each, group III and VI of thirteen each, while group IV had twelve animals. In all the groups ulceration was produced. Groups II to VI were injected with 1 c.c. (0.5 mg./c.c.) of the respective anticholinergic drugs, while group I received 1 c.c. of distilled water. All the injections were given intraperitoneally fifteen minutes before first injection of promethazine hydrochloride. The animals were sacrificed four hours after the first injection.

Histological technique.—Autopsy was performed on all the animals, gastric contents collected and the degree of ulceration graded, maximum score being hundred (Zaidi and Mukerji, *loc. cit.*). The index of ulceration was calculated according to the method of Pauls, Wick and McKay (1947). Tissue blocks were fixed, embedded in paraffin and cut at 5 μ . Sections were stained with hæmatoxylin and eosin.

Biochemical technique.—The gastric contents from each animal were centrifuged to remove food particles and after suitable dilution mucin was estimated (Dische, 1947). Free and total acidity using 0.01 N-NaOH and Topfer and phenolphthalein as indicators was titrated and peptic activity determined by Mett Tube method (Hawk *et al.*, 1947).

RESULTS.

Histopathology:

Gross appearance.—The naked-eye examination of the stomach *in situ* in groups I to VI showed in some animals ulceration of varying degree and extent in fundus and pylorus, while the mucosa of other animals had normal appearance. In group I, thirteen animals had ulceration and the rest two showed only congestion. In group II, all the animals, except one which had ulceration, were normal. In group III six animals showed complete protection from ulceration, while the remaining seven were ulcerated. Groups IV, V and VI had five, three and two animals out of twelve, fourteen and thirteen, respectively, which were completely protected from ulceration. Protected animals showed smooth outer surface and normal gastric mucosa. The degree of ulceration in control group I was maximum, while the lowest was noted in group II. In groups III, IV, V and VI the degree of ulceration varied. The gastric juice in the ulcerated animals was chocolate brown.

The degree and index of ulceration is shown in Table I and the Text-figure.

Microscopic appearance.—The ulcerated animals of groups I to VI showed varying degree of damage of the gastric mucosa. The superficial ulceration showed complete destruction of the mucosa (Plate XXI, fig. 1), while deep ulceration was accompanied by destruction of the muscularis mucosae, submucosa and muscular layers (Plate XXI, fig. 2). Deep ulcers were mostly present in group I. Ulceration was accompanied by marked congestion and diffuse infiltration with acute inflammatory cells mostly polymorphs. The protected animals showed no histological abnormality.

Biochemical findings.—The results of biochemical investigations are summarized in Table II and the Text-figure.

Experimental Peptic Ulceration.

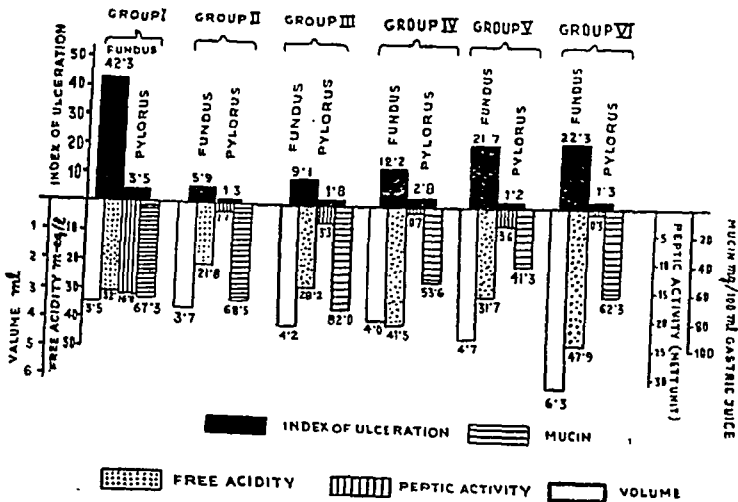
TABLE I.
Summary of results of the degree of gastric ulceration.

Group.	DEGREE OF ULCERATION:		INDEX OF ULCERATION*:	
	Fundus.	Pylorus.	Fundus.	Pylorus.
I	45.3	3.7	42.3	3.5
II	9.2	2.1	5.9	1.3
III	11.1	2.8	9.1	1.8
IV	18.3	4.2	12.2	2.8
V	25.4	1.4	21.7	1.2
VI	26.4	1.5	22.3	1.3

Group I histamine and promethazine hydrochloride, group II oxyphenonium bromide, group III atropine sulphate, group IV scopolamine methylbromide, group V pro-banthine and group VI esentil (ulceration was produced in groups II to VI by the same procedure as in group I).

$$\text{*Index of ulceration} = \frac{\text{Degree of ulceration} \times \text{per cent of group ulcerated}}{100}$$

TEXT-FIGURE.



Shows index of ulceration and mean values of biochemical investigations. Group I hitamine and promethazine hydrochloride, group II oxyphenonium bromide, group III atropine sulphate, group IV scopolamine methylbromide, group V pro-banthine and group VI esentil (ulceration was produced in groups II to VI by the same procedure as in group I).

Anticholinergic drugs used in the present experiments did not show a marked change in the volume of the gastric juice. Oxyphenonium bromide and atropine sulphate depressed free acidity, while other drugs had little or no effect on the acid content. The marked action of these drugs, however, was on peptic activity. With oxyphenonium bromide, scopolamine methylbromide and esentil no peptic activity could be detected in the gastric contents except in one animal each in the respective groups. Pro-banthine and atropine sulphate was also noted to have

depressant effect on the peptic activity. Under the present experimental conditions, the values of mucin in all the groups did not, however, substantially differ.

TABLE II.

Summary of results of investigation of gastric contents.

Group:	I	II	III	IV	V	VI
Number of animal:	15	14	13	12	14	13
Volume, c.c.	Mean 3.5 median 2.8 range 1.8-8.3	Mean 3.7 median 2.7 range 0.8-7.7	Mean 4.2 median 3.4 range 1.8-8.8	Mean 4.0 median 3.8 range 1.8-7.3	Mean 4.7 median 3.7 range 2.2-8.3	Mean 6.3 median 4.3 range 1.8-10.8
Free acidity m.eq./l	Mean 32.0 median 34 range 0-92	Mean 21.8 median 10 range 0-62	Mean 28.2 median 13 range 0-88	Mean 41.5 median 33 range 10-92	Mean 31.7 median 53 range 0-92	Mean 47.9 median 56 range 0-110
Total acidity m.eq./l	Mean 52.2 median 51 range 5-114	Mean 41.0 median 34 range 4-90	Mean 49.0 median 40 range 6-115	Mean 62.9 median 62 range 24-114	Mean 41.5 median 67 range 6-116	Mean 65.9 median 60 range 20-138
Peptic activity Mett Unit	Mean 16.8 median 16 range 0-92	Mean 1.1 median 0 range 0-16	Mean 3.3 median 4 range 0-8	Mean 0.7 median 0 range 0-6	Mean 3.6 median 0 range 0-23	Mean 0.3 median 0 range 0-4
Mucin mg. per cent	Mean 67.3 median 42 range 21-168	Mean 68.5 median 74 range 0-189	Mean 82.0 median 63 range 0-220	Mean 53.6 median 45 range 11-126	Mean 41.3 median 36 range 15-172	Mean 62.3 median 57 range 32-105

Group I histamine and promethazine hydrochloride, group II oxyphenonium bromide, group III atropine sulphate, group IV scopolamine methylbromide, group V pro-banthine and group VI esential (ulceration was produced in groups II to VI by the same procedure as used in group I).

DISCUSSION.

Anticholinergic drugs studied for their anti-ulcer activity in pylorus ligated rats had a preventive action against rumenal ulceration (Shay *et al.*, 1945; Little *et al.*, 1947, Komarov *et al.*, 1950; and Barrett *et al.*, 1953). Our experiments in histamine-induced ulceration in guinea-pigs confirm the ulcer preventive action of anticholinergic drugs. The protection from ulceration by these drugs was of varying degree, maximum being achieved by oxyphenonium bromide followed by atropine sulphate, scopolamine methyl-bromide, pro-banthine and esential in that order. The mechanism of protection from ulceration is, however, not clear.

Plummer *et al.* (1953) noted that the volume of gastric secretion in the Pavlov dogs induced by histamine was not inhibited by anticholinergic drug oxyphenonium bromide. Barrett *et al.* (*loc. cit.*), on the other hand, showed that it consistently suppressed the gastric secretion in rats evoked by the subcutaneous injections of histamine. Atropine was also found to be an effective inhibitor of gastric secretion produced by subcutaneous injections of small doses of histamine but increased dose of histamine diminished its inhibitory effect (Gray, 1937). Kirsner and Palmer (1953) noted that scopolamine methylbromide inhibited gastric

Experimental Peptic Ulceration.

secretion by decreasing volume and Lichstein *et al.* (*loc. cit.*) noted the same effect with pro-banthine. On the basis of these observations Barrett *et al.* (*loc. cit.*) concluded that the success in the prevention of experimental peptic ulceration may be due to the direct effect of anticholinergic drugs in the reduction of the volume of gastric secretion. In the present experiments, however, the ulcer prevention by the action of anticholinergic drugs does not appear to be by the suppression of the volume of gastric juice. It is possible that large doses of these drugs may have lowered gastric secretion, but Kirsner, Levin and Palmer (*loc. cit.*) noted that large doses induced systemic manifestations of parasympathetic inhibition which constituted a major disadvantage and necessitated the discontinuance of the drug in the treatment of the peptic ulcer.

Rowen *et al.* (1953) noted little change in acid concentration with oxyphenonium bromide. Barrett *et al.* (*loc. cit.*) showed statistically significant reduction in acidity with atropine. Rider *et al.* (*loc. cit.*) observed that scopolamine methylbromide did not consistently suppress free acidity. Kirsner and Palmer (*loc. cit.*) noted suppression of free acidity with pro-banthine. The effect of esential on free acidity is inconclusive. From the results of these studies it is apparent that differing conclusions as regards the effect of these drugs on acidity have been reached. Anticholinergic drugs in the present experiments did not, however, bring about marked change in free acidity. The exception has been oxyphenonium bromide and atropine sulphate where there was a suggestion of depression in the acid content. In the mechanism of the prevention of ulceration by anticholinergic drugs it, therefore, appears that suppression of free acidity is not an important factor and that mucous also does not play any part as no rise in its values were noted in any group.

The question, therefore, of the rôle of peptic activity in relation to peptic ulceration assumes significance. LeVeen (1947) showed that increased peptic activity may cause ulceration and Singh, Zaidi and Balkrishna (*loc. cit.*) demonstrated in pylorus ligated rats positive relationship between increased peptic activity and ulceration. The rôle of peptic activity in acute histamine-induced ulceration in guinea-pigs is not clear (Zaidi and Mukerji, *loc. cit.*). The present investigations, however, demonstrate a marked inhibition of peptic activity by oxyphenonium bromide, scopolamine methylbromide and esential, while pro-banthine and atropine sulphate to a lesser degree. This inhibition may be due to their blocking action on the vagus nerves supplying the peptic cells. These experiments indicate that this effect on peptic activity is related to the degree of prevention from ulceration.

Kirsner and Palmer (*loc. cit.*) suggested that 'the ideal gastric antisecretory agent applicable to the treatment of peptic ulcer may be characterized as a safely administered compound that consistently inhibits the output of hydrochloric acid for long periods after oral ingestion without development of tolerance and with minimal or no side-effects' and Lichstein *et al.* (*loc. cit.*) observed that anticholinergic group of drugs may closely achieve the ideal of an anti-secretory drug. The present study of the anticholinergic drugs afford some useful data as regards prevention from ulceration in experimental animals, but how far these drugs will be of use as 'prophylaxis' and as a cure in peptic-ulcer therapy need extensive and prolonged clinical investigation.

SUMMARY.

1. Anticholinergic drugs atropine sulphate, oxyphenonium bromide, scopolamine methylbromide, pro-banthine and esentil have been studied for their anti-ulcer activity in guinea-pigs in which ulceration was produced with massive doses of histamine protected against its toxic effects by an antihistaminic, promethazine hydrochloride.

2. The maximum protection from ulceration was achieved by oxyphenonium bromide followed by atropine sulphate, scopolamine methylbromide, pro-banthine and esentil in that order. The mechanism of protection from ulceration has been discussed.

3. Anticholinergic drugs used in the present investigations do not appear to cause an increase of mucous or consistently suppress the volume or free acidity of the gastric contents. There is, however, a marked inhibition of peptic activity. The degree of prevention of ulceration by anticholinergic drugs is related to the inhibition of peptic activity.

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CHEMICAL CHARACTERIZATION OF CHAKRANINE.

(a) *Micro-analysis, UV and IR spectra.*—

Chakranine conforms to the formula $C_{21}H_{24}O_4NCl$:

	C	H	N	Cl
found	64.24	6.58	3.64	9.27
requires	64.60	6.60	3.60	9.17

A methoxy group is indicated by Zeisel's technique and an active hydrogen atom by Zerewitinoff.

UV and IR spectra show that this compound contains a benzenoid system and an NH-group. The absence of any carbonyl and hydroxyl groups is also indicated.

(b) *Properties.*—Chakranine is highly soluble in water, ethyl alcohol and methyl alcohol, moderately soluble in chloroform, sparingly soluble in acetone and ethyl acetate and practically insoluble in petroleum ether, sulphuric ether and benzene. Its water solution is feebly basic to litmus.

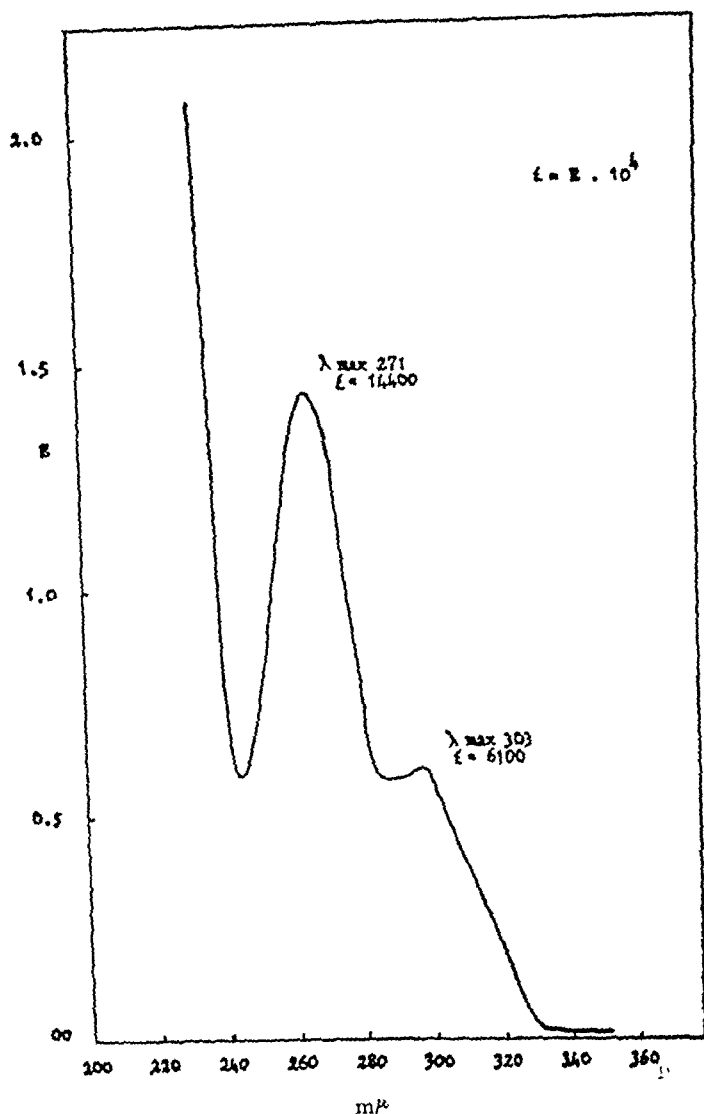
It gives a voluminous precipitate with most of the alkaloidal reagents. With $FeCl_3$, it does not develop any blue or green colouration but the colour of the mixture slowly deepens, finally turning into a beautiful wine-red. With $NaNO_2$ and H_2SO_4 , it gives a yellow nitroso compound which answers positive to the Liebermann reaction, giving a deep greenish-blue colour, thus indicating the presence of a secondary amino group. With $AgNO_3$, it gives a voluminous white precipitate which is insoluble in nitric acid. With concentrated H_2SO_4 , there is no colouration in the cold, but on warming the solution turns violetish-pink; the cold solution on the addition of a few specks of potassium dichromate turns bluish violet immediately. With HNO_3 , an intense blood red colouration is immediately produced. With bromine-water, it gives a red solution, the colour of which is discharged on addition of a little sodium bisulphite; on addition of ammonia to this hot solution a violet colour develops. With potassium ferrocyanide and sulphuric acid, it gives rise to a greenish-blue colouration slowly. Sulphomolybdic acid gives a green colour in the cold which turns yellowish-brown on heating. Neutral potassium permanganate solution is decolourized immediately. Chakranine reduces Tollen's reagent only slowly in the cold, but quickly in hot. It does not develop any characteristic colouration with para-dimethylaminobenzaldehyde. Adamkiewicz reaction was also negative.

(c) *Derivatives of Chakranine.*—A picrate (m.p. 201-203°C.), an acetate (m.p. 253°C.) and a sulphate (m.p. 240°C.) were obtained in a pure crystalline form in the following manner:

(a) *Chakranine picrate.*—190 mg. of chakranine dissolved in alcohol were made to react overnight at room temperature with 115 mg. of picric acid dissolved in absolute alcohol. Thereafter, the solvent was removed under reduced pressure, and the amorphous residue successively washed with cold acetone and water in order to free it from any unreacted picric acid and chakranine. It crystallized from methanol-water mixture as yellow, prismatic needles, m.p. 210 to 203°C.

(b) *Chakranine acetate.*—250 mg. of chakranine were intimately mixed with 500 mg. of fused sodium acetate by grinding in a mortar, and then added in small

GRAPH 1.

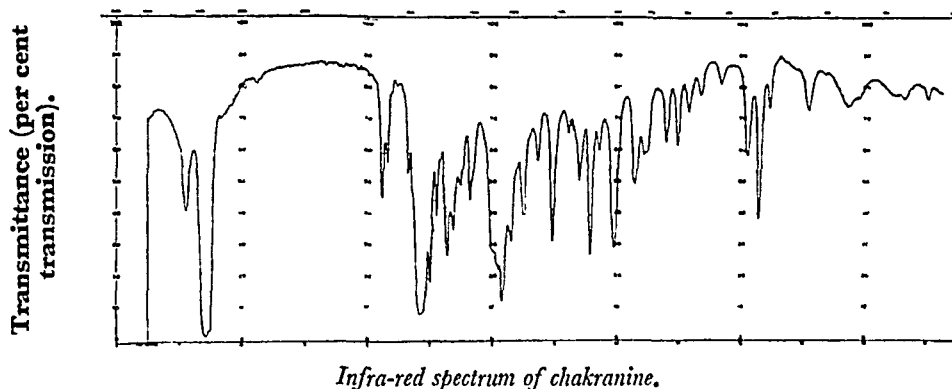


Ultraviolet spectrum of chakranine.

loss to 20 c.c. of acetic anhydride. The reaction mixture was carefully heated at a constant boiling temperature for 4 hours in a test-tube fitted with an air-condenser. At the end of this period, the reaction mixture was cooled, diluted with twice the volume of water and extracted with chloroform. The $CHCl_3$ -layer was worked up in conventional manner. The acetate finally crystallized from a chloroform-ethanol-acetone mixture, in the form of small prisms, melting at $253^\circ C$.

(c) *Chakranine sulphate*.—To 130 mg. of chakranine, dissolved in absolute alcohol, was added a slight excess of sulphuric acid solution in alcohol. The

GRAPH 2.



reaction-mixture was allowed to stand overnight at room temperature. Thereafter, the solvent was allowed to evaporate slowly at $50^{\circ}\text{C}.$, when it left behind a syrupy liquid. This was first washed with sulphuric ether, and the ether washings rejected. The semi-dry residue was then kneaded with dry acetone, whereupon it slowly developed into colourless prisms, m.p. $240^{\circ}\text{C}.$

PHARMACOLOGICAL CHARACTERIZATION OF CHAKRANINE.

(a) *Acute toxicity*.—This was determined in mice, average weight 18 g. to 21 g., (i) by the intravenous route, and (ii) by oral administration.

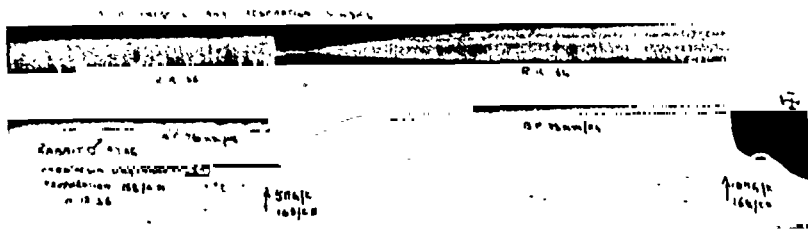
Intravenously, all animals receiving a dose of 2.5 mg./kg. and above (5 mg., 10 mg., and 100 mg.) succumbed instantaneously to within a period of one-half hour with marked dyspnoea, muscular spasms, clonic to tetanic in character. With 2 mg./kg. dose, there was only 20 per cent mortality during the period of 10 days of observation. LD_{50} worked out as 0.0022 g./kg.

When tested by mouth the results were entirely different. With a dose of 100 mg./kg. there was no mortality and no toxic manifestations during the 10 days of observation. This was surprising in view of the high water solubility of chakranine.

(b) *Blood pressure and respiration*.—In rabbits anaesthetized with urethane (1.5 g./kg.) the jugular vein and trachea were canulated and blood pressure and respiratory movements recorded in the conventional manner. With 0.5 mg./kg. there was approximately 40 per cent depression in blood pressure lasting for 3 minutes. This was associated with slight respiratory arrest followed by nearly 20 per cent increase in the respiratory rate. With ten-fold increase in dose (5 mg./kg.), whereas the blood pressure depression was the same as with 0.5 mg./kg., respiration was far more affected, there being marked respiratory arrest (Text-figure 1). After 4 to 5 minutes apnoea, however, respiratory rate increased by approximately 20 per cent. With 10 mg./kg. dose there was a sudden and rapid depression of blood pressure along with complete and lasting respiratory arrest resulting in the death of the animal.

(c) *Isolated frog heart* (*Rana tigrina*).—There was no action on this muscle in dose concentration of 10^{-6} , 10^{-5} and 10^{-4} . With 10^{-3} there was approximately 10 per cent positive inotropism, reversible on washing. With 1 per cent solution

TEXT-FIGURE 1.



Effect of chakranine on respiration and blood pressure of urethane-anæsthetized rabbit.

of chakranine (10^{-2}) nearly 50 per cent positive inotropism and marked negative chronotropism were observed.

(d) *Direct action and antagonisms.*—(i) On isolated intestine of rabbit there was no action in dosage varying from 10^{-7} to 10^{-4} and no antagonism against acetylcholine (dilution 5×10^{-7}) and barium chloride (dilution 2×10^{-4}).

(ii) On isolated intestine of guinea-pig, there was, however, 100 per cent antagonism against histamine (dilution 5×10^{-8}) with a chakranine dose concentration of 10^{-4} .

(iii) On isolated uterus of guinea-pig, there was marked tonic contraction of uterus, reversible on prolonged washing.

(e) *Action on rabbit intestine in situ.*—Studies were conducted on urethane anæsthetized rabbits. A specially constructed socket fitted with a glass cylinder was used to suspend the intestinal loop on to a frontal writing lever and recordings taken on the kymograph. The peritoneum was filled with tyrode solution maintained at 40°C . The test substance was administered to the animal through the cannulated jugular vein.

Chakranine was administered intravenously in dosage of 0.5 mg., 1 mg. and 5 mg./kg. The results presented in Text-figure 2, already indicate with 1 mg. some increase in intestinal persistalsis which is well marked with 5 mg. This was unexpected in view of the previous experience with isolated rabbit intestine wherein a dose of 10 mg. was found to be ineffective in enhancing the contraction of intestinal musculature. To get clarification of this anomalous result, it was decided to administer as much as 100 mg. of chakranine orally, a dose found to be non-toxic in previous studies. The intestinal movements were recorded thereafter for a period of an hour and a half. No contraction of intestinal loop took place (*vide* Text-figure 2).

Limited as these pharmacological studies are, nevertheless, some points of interest arise therefrom. It is difficult to explain the underlying cause of marked differences in acute toxicity of parentally and orally administered chakranine, an alkaloid that is highly water soluble. *In vitro* observations on pH variations do not account for this discrepancy; chakranine forms a stable solution with 10 per cent NaOH. The possibility of precipitation under alkaline condition in the intestinal

TEXT-FIGURE 2.



Rabbit intestine in situ: Action of chakranine given intravenously (0.5 mg, 1.0 mg, and 5.0 mg./kg.) and by mouth (100 mg./kg.)

canal is, therefore, discounted. The inference is that for some reason yet unknown this alkaloid is not absorbed when given by mouth.

The physiological activity of chakranine is characterized by a ganglion blocking effect, selectively exercised against the autonomous nerve pathways of respiratory mechanism. This is of a low order and temporary in character, the receptor site, in all probability, being the presynaptic nerve fibril which are paralysed for a short period of time.

By direct application, in a comparatively high dose (10^{-4}) the smooth muscle of the isolated uterus is stimulated, whereas a similar dose exercises a significant histaminolytic action on the isolated intestine of guinea-pig. In contrast, the isolated intestine of rabbit is unaffected, but the same organ *in situ* responds with a marked contraction in dose concentration of 5 mg./kg. intravenously, whereas a twenty-fold increase of chakranine by mouth, under identical conditions of experimentation, is without any effect. It is justifiable to assume from these results that this alkaloid is, probably, excreted through the intestinal canal. Further pharmacological investigations are in progress.

ANTIBACTERIAL ACTIVITY OF CHAKRANINE.

This was determined preliminarily by following the ditch-plate technique and thereafter assessed quantitatively by the serial dilution method. The results are presented in Tables I and II.

TABLE I.

Antibacterial spectrum of chakranine and iso-aristolochic acid.
(Ditch-plate method.)

Test organisms.	RESULTS:	
	Chakranine	Iso-aristolochic acid.
<i>M. pyogenes</i> var. <i>aureus</i> (FDA 209) ..	+	+
<i>D. pneumoniae</i> Type II-NCTC 7466 ..	—	—
<i>St. pyogenes</i> (ATCC 8668) ..	+	—
<i>St. viridans</i> I (NCTC 3166) ..	—	—
<i>E. coli</i> var. <i>communis</i> (NCIC 86) ..	—	—
<i>V. comma</i> Inaba (PI 1800/1) ..	—	—
<i>Sh. dysenteriae</i> var. <i>Shiga</i> (MRC 2101) ..	—	—

TABLE II.

Antibacterial activity of chakranine.

Test organisms.	CONCENTRATION OF DRUG IN MG./C.C.:					
	1.0	0.5	0.3	0.2	0.1	0 (control).
<i>M. pyogenes</i> var. <i>aureus</i> (FDA 209)	N	N	S	C	C	C
<i>St. pyogenes</i> (ATCC 8668)	N	N	N	S	C	C

N=No growth; S=Scanty growth; C=Confluent growth.

For serial dilution, 10 c.c. blood broth, containing 10 per cent rabbit blood was used as a medium for *Str. pyogenes* and an equal volume of nutrient broth for *M. pyogenes* var. *aureus*. 0.1 c.c. of an 18-hour old culture was the test inoculum. Growth or its inhibition was assessed by plating out 0.01 c.c. of the test mixture after 24 hours incubation at 37° C.

It is evident from the results obtained that the antibacterial action of chakranine is limited to only two of the Gram-positive micro-organism and the degree of this activity is of a low order.

In view of the reputation which *Bragantia Wallichii* enjoys in the treatment of intestinal infections, and specially that of cholera, iso-aristolochic acid and the chakranine-free alcoholic extract were subjected to careful antibacterial analysis. The results were, however, uniformly negative, particularly against *Vibrio cholera* and the Gram-negative micro-organisms.

SUMMARY.

1. The isolation of a crystalline alkaloidal principle from the roots of *Bragantia Wallichii* R.Br., has been described. This new chemical compound has been designated as 'Chakranine'.

2. Some of the chemical, pharmacological and antibacterial properties of chakranine have been studied.

3. The experimental data presented here does not lend support to the reputation which this plant material enjoys in the treatment of intestinal infections and other clinical disorders.

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HYPOTHALAMIC CONTROL OVER WATER CONSUMPTION IN THE RAT*.

BY

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THE rôle of hypothalamus in the regulation of food intake has been well established. Both hyperphagia and aphagia have been produced after hypothalamic lesions as well as stimulations in unanæsthetized animals (Hetherington and Ranson, 1940 and 1942; Brobeck, Tepperman and Long, 1943; Wheatley, 1944; Kennedy, 1950; Anand and Brobeck, 1951 and 1951a; Anand and Dua, 1955; and Anand, Dua and Shoenberg, 1955).

Stevenson and others (1949 and 1950) observed that ventromedial lesions in the rat, which produce hyperphagia and obesity, also produce a reduced water/food intake ratio, which they ascribed to a 'deficit in the normal mechanism responsible for thirst and its alleviation'. They called it 'hypothalamic hypodipsia'. Anand and Brobeck (1951a) reported that the rats which become aphagic after lateral hypothalamic lesions, also show complete adipsia. Anderson (1952) further confirmed the hypothalamic influence by the induction of thirst in goats upon injection of hypertonic saline into the medial hypothalamus, and Anderson and McCann (1955 and 1956) later located their 'drinking area' in the region of dorsal hypothalamus, by stimulation studies in goats, and producing electrolytic lesions in dogs. Greer (1955) has reported a case of a rat showing 'violent drinking activity' upon electrical stimulation of lateral hypothalamus. Montemurro and Stevenson (1955/56) have now reported that the low water/food intake ratio after medial hypothalamic lesions was due to a dramatic increase in food consumption without any significant increase in water intake; on the other hand, after very tiny lesions in lateral hypothalamus, which were too small to decrease the food intake, the food intake remained about normal but there was a

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reduction in water intake. They have, therefore, inferred that hypothalamic area responsible for hypodipsia 'which is apparently important in the normal regulation of water intake' is situated in the same region as 'feeding centre'. More recently, Morrison and Mayer (1957) have reported that rats made aphagic after lesions in the 'feeding centre' are also completely adipsic, and this adipsia is a primary effect of lateral hypothalamic lesions.

The purpose of the present study was, therefore, to locate more precisely the hypothalamic regions producing 'hypodipsia', and thus try to clarify the hypothalamic regions controlling water consumption.

MATERIALS AND METHODS.

Forty albino rats of the strain being bred at Kasauli, weighing between 150 and 175 g., were used for this study. Thirty out of these survived up to the completion of the study, the remaining dying in the post-operative period. These were housed in individual cages at an environmental temperature of approximately 85°F. They were fed *ad libitum* on a high carbohydrate diet with necessary amounts of vitamins and salts. Water was given through separate graduated bottles. A daily record of their food and water intake was kept both before and after the operation.

Bilateral electrolytic lesions were placed in different hypothalamic regions with the aid of Horsley-Clark stereotaxic instrument. The hypothalamus was divided into ten small discrete regions, and in each rat bilateral lesions were placed in one of these regions. The co-ordinates used for placing lesions and the hypothalamic regions where such lesions were produced are shown in Plate XXII, fig. 1. All these lesions were placed 1 mm. above the base of brain.

Intraperitoneal sodium evipal (12 mg./100 g. body-weight) was used for anaesthesia. The lesions were made with a unipolar electrode, using a direct current of 1.5 ma. flowing for 15 seconds. After over a month these rats were sacrificed and the true location and extent of the lesions determined histologically.

The food and water intake values of these rats recorded for a period of 30 days before operation have been used as controls, for comparison with the food and water intake values of the same rats recorded for 30 days after the operation.

RESULTS.

The averages of food and water intake, and water/food ratio of the 30 rats (which survived the post-operative period) worked out for 30 days following the hypothalamic lesions, have been compared with the averages of these figures worked out for 30 days prior to the operation in the Table. It will be seen that all the rats after hypothalamic lesions produced some lowering of water/food ratio, indicating that these rats did not drink as much water per gramme of food eaten as they did before the lesions.

Rats after lesions placed at the co-ordinates of $A_6 R_2 L_2$ ('feeding centre'—Anand and Brobeck, 1951 and 1951a) could not survive long enough to be included in the Table. It has already been reported by us that such lesions, in addition to

producing complete aphagia, also produce complete adipsia for the duration of survival of the animal.

TABLE.

Average of food and water intakes of rats after hypothalamic lesions, compared with pre-operative period.

Location of lesions*.	Number of rats.	Duration, days.	Food.	Water.	Water/Food.
			g./rat/day.	c.c./rat/day.	c.c./g./day.
A ₈ R ₁ L ₁ ..	3	30	†11·21±†2·00	18·13±0·24	1·61
A ₈ R ₂ L ₂ ..	4	30	†10·83±1·63	15·38±3·68	1·42
A ₇ R ₁ L ₁ ..	3	30	†11·92±0·93	18·64±3·67	1·56
A ₇ R ₂ L ₂ ..	4	30	†10·17±1·89	†13·05±2·59	1·28
A ₆ R ₁ L ₁ ..	4	30	†12·10±3·7	†13·22±3·96	1·09
A ₆ R ₂ L ₂ ..	2		D I E D		
A ₅ R ₁ L ₁ ..	4	30	†10·82±1·54	16·21±2·26	1·49
A ₅ R ₂ L ₂ ..	3	30	†10·58±2·4	16·18±3·93	1·53
A ₄ R ₁ L ₁ ..	2	30	†11·93±0·74	21·00±3·55	1·76
A ₄ R ₂ L ₂ ..	3	30	†11·73±0·50	16·08±2·72	1·37
Control (before lesions) ..	25	30	9·05±0·81	16·95±3·49	1·87

*Horsley-Clarke Co-ordinates (see Fig. 1).

†Standard error of mean.

‡Statistically significant change from control.

The water/food ratios were significantly lower than those of controls only after lesions at the co-ordinates of A₆ R₁ L₁ (Plate XXII, fig. 2) and A₇ R₂ L₂ (Plate XXIII, fig. 3) i.e. in the regions which are 1 mm. anterior and 1 mm. medial to the 'feeding centre', and the total daily water intake also was significantly lower after lesions in these two regions, as compared with controls. This decrease in daily water consumption was accompanied by a significant increase in the daily food intake after lesions at A₆ R₁ L₁ (ventromedial nuclei—'satiety centre'), while after lesions at A₇ R₂ L₂, the food intake did not show a significant change. That is why, in spite of the fact that water intake decreases almost equally after both these lesions, the water/food ratio is decreased more after A₆ R₁ L₁ lesions than after A₇ R₂ L₂ lesions.

After lesions in the other hypothalamic regions, the slightly decreased water/food ratio is not due to any significant change in the water consumption, but is due to some elevation in food consumption with little change in water intake.

It appears, then, that lesions in the 'feeding centre' produce complete adipsia alongwith complete aphagia, but after lesions in other hypothalamic regions the total water consumption does not just follow the changes in the food intake. If the lesions are near the 'feeding centre' (1 mm. anterior or medial), hypodipsia is produced in spite of the fact whether food intake changes or not. If the lesions are further removed, there is no significant change in the water consumption, in spite of any change in food intake.

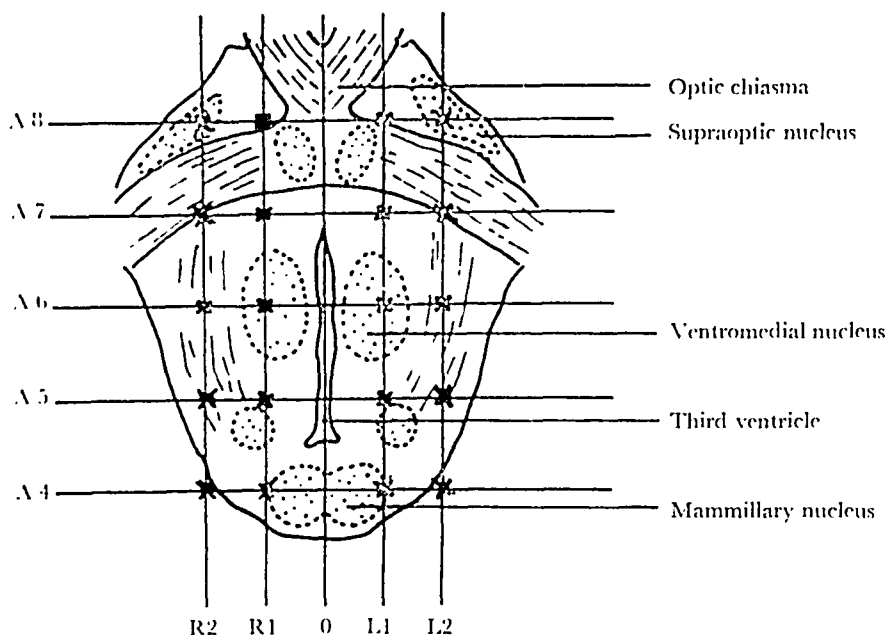


FIG. 1. Diagram of the hypothalamus of the rat showing the Horsely-Clarke co-ordinates employed. The line labelled O represents the mid-sagittal plane of the brain. Lines labelled R₁, R₂, and L₁, L₂ represent planes 1 mm. on either side of the mid-sagittal plane. Lines labelled A₄, A₅, A₆, A₇, and A₈ represent planes anterior to the zero frontal plane which runs through the external auditory meatus. The crosses indicate different positions of the lesions produced.



FIG. 2. Photomicrograph of section through the diencephalon of rat 10, showing small bilateral lesions in the medial hypothalamus in the plane of the ventromedial nuclei (A₆R₁L₁). These lesions are 1 mm. medial to the 'feeding' centres.



FIG. 3. Photomicrograph of section through the diencephalon of rat 13, showing small bilateral lesions in the lateral hypothalamus in the plane of the anterior hypothalamus ($A_7R_2L_2$). These lesions are 1 mm. anterior to the 'feeding centres'.

DISCUSSION.

Although the regulation of renal water excretion has been clearly associated with the supraoptic nuclei in the anterior hypothalamus (Verney, 1947), the regulation of water intake has not been accurately localized.

In the normal rat, when the composition of the diet and other conditions are kept constant, the *ad libitum* water consumption varies more or less directly with the amount of food eaten, whether this be varied by increasing the food requirement or by restricting its supply. After hypothalamic lesions, the animals failed to show this correlation of water with food intake. This has also been observed by Montemurro and Stevenson (*loc. cit.*). Thus, the complete adipsia produced after lesions in the lateral hypothalamic regions ('feeding centre') may not be explainable as just due to complete absence of food intake. Montemurro and Stevenson showed that if the lesions produced in this region are very tiny, they do not produce any change in food intake, but lead to hypodipsia. Greer (*loc. cit.*), on the other hand, observed in one rat violent drinking activity on electrical stimulation of this region. Morrison and Mayer (*loc. cit.*) have also suggested that the adipsia occurring alongwith aphagia is a primary effect of lateral hypothalamic lesions.

We have also observed that if the hypothalamic lesions are produced near about the 'feeding centre' (1 mm. medial or anterior), they lead to lowered water consumption (hypodipsia), in spite of whether the food intake increases or remains normal. Montemurro and Stevenson (*loc. cit.*) had observed that medial hypothalamic lesions do not decrease daily water consumption, but only lower the water/food ratio by increasing food intake without a concomitant rise in water consumption.

Lesions further removed from here do not significantly change water consumption, whether food intake increases or not. Small lesions in the region of supraoptic nuclei also did not increase urinary excretion and water intake (diabetes insipidus).

It is thus evident that the hypothalamic region controlling food intake ('feeding centre') and its surrounding regions, together constitute a neural mechanism responsible for thirst and its alleviation. As this mechanism is not restricted to a small spot but occupies a comparatively bigger area, different workers have located it in slightly differing regions, which all could be included in this area. Montemurro and Stevenson (*loc. cit.*) and Morrison and Mayer (*loc. cit.*) have located their 'thirst centre' in the same area as 'feeding centre', Stevenson (*loc. cit.*) located it in medial hypothalamus, and Anderson and McCann (1955 and 1956) located their 'drinking area' in the region of dorsal hypothalamus.

There is little doubt that a close relation between water regulation and energy metabolism exists. It seems possible that an integrated regulation of both these essential functions is situated in the same area within the hypothalamus.

The exact nature of the stimulus to this area and the mechanism by which it responds to produce a physiological effect require further investigation. Evidence has been presented in favour of anosmotic stimulus, as Anderson (*loc. cit.*) produced

immediate polydipsia in the conscious goat by intrahypothalamic injections of hypertonic saline.

SUMMARY.

1. With the use of the Horsley-Clarke stereotaxic instrument, electrolytic lesions were placed at various bilateral loci in the hypothalamus of the albino rats. Variations in water and food intake produced as a result of these were compared with controls.

2. After hypothalamic lesions the rats fail to show the correlation of water with food intake, observed in normal animals.

3. Lesions in the lateral hypothalamus ('feeding centre') in addition to complete aphagia also produce complete adipisia. Lesions near about this region (1 mm. medial or anterior) produce hypodipsia irrespective of increase in food intake. Lesions further removed from this region, do not significantly change water intake in spite of changes in food intake.

4. It is concluded that the hypothalamic region controlling food intake and its surrounding regions, together constitute a neural mechanism responsible for thirst and its alleviation.

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NICOTINIC ACID-TRYPTOPHAN METABOLISM IN MAN.

BY

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THE increased urinary excretions of nicotinic acid, quinolinic acid, N'-methylnicotinamide and 6-pyridone, after the administration of tryptophan in man, have been reported by several workers (Perlzweig *et al.*, 1947; Sarett and Goldsmith, 1950; Rosenthol, Goldsmith and Sarett, 1953; Goldsmith *et al.*, 1952). Studies made by Kallio and Berge (1949) have indicated that kynurenin probably is also a precursor of nicotinic acid. Other metabolites of tryptophan, such as anthranilic acid and 3-hydroxyanthranilic acid, etc., have also been demonstrated in urine (Reddi and Kodicek, 1953; Dalgeleish, 1955; Brown and Price, 1956 and 1956 *a*). Studies on the urinary excretions of different metabolites of nicotinic acid and tryptophan, however, as mentioned above, after a dose of tryptophan have not been undertaken in the same individual so far which prompted the authors to undertake the present investigations.

EXPERIMENTAL.

Ten adult males from the research students of the laboratory were selected for the study. Their ages varied between 19 and 26 years. They ate their usual diet but the quantity and quality of food did not vary during the experimental period. The 24-hour urine was collected in a bottle containing 10 c.c. toluene and complete collection of urine was checked by estimation of creatinine. Normal urinary excretions of different metabolites were determined for two consecutive days. At the end of second day's collection they were fed 5 g. of DL-tryptophan which was again repeated after 24 hours and 24-hour urine were collected daily for three subsequent days. Nicotinic acid and amide, quinolinic acid, N'-methylnicotinamide, 6-pyridone, tryptophan, kynurenin, anthranilic acid and 3-hydroxyanthranilic acid were estimated by the methods mentioned as follows.

Estimation of nicotinic acid.—Nicotinic acid was estimated according to the method of Banerjee, Ghosh and Bhattacharya (1948) as modified by Nand and Banerjee (1949) in our Laboratory.

Estimation of quinolinic acid.—Quinolinic acid was estimated by the method of Sarett (1951).

Estimation of N'-methylnicotinamide.—N'MN was estimated by the method of Carpenter and Kodicek (1950), slightly modified by us to avoid interference with riboflavin.

Estimation of 6-pyridone.—6-pyridone was estimated by the method of Rosen, Perlzweig and Leder (1949).

Estimation of tryptophan, kynurenin, anthranilic acid and 3-hydroxyanthranilic acid.—These substances were estimated by paper chromatography according to the method of Dalgeleish (*loc. cit.*) suitably modified to give fairly quantitative results. Tryptophan metabolites in urine were adsorbed on de-activated charcoal, eluted with aqueous phenol, the eluate concentrated, fractionated by paper chromatography, sprayed with Ehrlich's reagent to develop colour and the colour estimated by photo-electric densitometer.

Recovery experiments undertaken indicated that the method employed is fairly quantitative as 88 to 96 per cent of the substances added to urine samples could be recovered.

The results of the different analyses are given in the Table:—

TABLE.

Twenty-four-hour urinary excretion of metabolites of nicotinic acid and tryptophan in mg. in normal men fed tryptophan for two consecutive days.

(Average excretions of 10 men.)

	Before feeding tryptophan.	AFTER FEEDING 5 G. DL-TRYPTOPHAN FOR 2 CONSECUTIVE DAYS:			
		1 Day.	2 Day.	3 Day.	4 Day.
Nicotinic acid and amide ..	4.78±.25*	7.63±.47	9.46±.52	7.76±.44	5.96±.47
Quinolinic acid ..	4.12±.55	10.7 ±.45	16.2 ±.71	8.65±.71	6.93±.22
N'MN ..	6.49±.53	13.32±1.03	22.4 ±.109	15.3 ±.75	9.38±.43
6-Pyridone ..	4.97±.94	34.3 ±3.93	46.9 ±.332	34.8 ±3.16	23.6 ±1.7
Tryptophan ..	61.1 ±3.56	1048 ±34.6	1481±63.2	386±20.2	148.7 ±7
Kynurenin ..	Absent	250.5±15.6	295.7±23.7	Absent	Absent
Anthranilic acid ..	Absent	Absent	Absent	Absent	Absent
3-Hydroxy-anthranilic acid ..	Absent	161.5±13.2	220.2±12.8	Absent	Absent

*Mean±Standard error.

DISCUSSION.

Normal men excreted in their urine nicotinic acid and amide, quinolinic acid, N'-methylnicotinamide, and 6-pyridone. All these substances were excreted in increased amounts after feeding DL-tryptophan.

Using Dalgeleish (*loc. cit.*) method of paper chromatographic analysis of the metabolites of urine, developing chromatograms with Ehrlich's reagent and reading the intensity of chromatogram by photo-electric densitometer, it has been concluded that this method is quite suitable for quantitative estimations of tryptophan, kynurenin, anthranilic acid and 3-hydroxyanthranilic acid. The average daily excretion of tryptophan in undosed subjects was 61.1 mg. These figures were higher than the figures reported by Frankl and Dunn (1947) who determined tryptophan microbiologically and lower than the figures reported by Albanese and Frankston (1945) who had used chemical method. We have estimated tryptophan by chromatography which is superior to chemical method. The urine might have contained some D-tryptophan which is not metabolized in the body and so our figures were higher than those reported by microbiological procedure. Urinary excretion of tryptophan reached the highest level on the day following, second dose of tryptophan; this was also excreted in increased amounts in urine, but considerably diminished on the third day and came to normal level on the fourth day.

Normal men did not excrete kynurenin, anthranilic acid and 3-hydroxyanthranilic acid in urine. After tryptophan feedings urine contained on the immediately following day considerable amounts of kynurenin and 3-hydroxyanthranilic acid. These substances disappeared on subsequent days. Urine did not contain any anthranilic acid even after tryptophan feeding. Reddi and Kodicek (*loc. cit.*), with the help of paper chromatography, have detected kynurenic acid, 3-hydroxyanthranilic acid, kynurenin and tryptophan after tryptophan dosing. They have not mentioned the urinary excretion of any tryptophan metabolite before tryptophan feeding. Brown and Price (1956a) have determined anthranilic acid glucuronide, O-aminohippuric acid, anthranilic acid, acetylkynurenin and kynurenin in human urine by column chromatography. Their findings agree with us in respect to absence of anthranilic acid in urine both before and after tryptophan administration. They could, however, detect kynurenin in undosed human urine which we could not by our method of paper chromatography. According to Brown and Price (1956a), 24-hour urine contained roughly 1.4 micromoles of kynurenin which might have been too low to be detected by our method. Of the different metabolites of nicotinic acid excreted in urine the excretion of 6-pyridone was maximum after the feeding of tryptophan.

The average tryptophan content of the daily diet of individuals was 3g. as determined microbiologically in our laboratory. With the amount of tryptophan in the diet urine did not contain any kynurenin and 3-hydroxyanthranilic acid which indicated that either tryptophan was metabolized beyond the stages or assimilated by the tissues. When load doses of 5 g. of DL-tryptophan were orally administered, kynurenin and 3-hydroxyanthranilic acid appeared in urine only on days following feeding, indicating that these metabolites of tryptophan could not be completely metabolized into either nicotinic acid or carbon dioxide and water.

SUMMARY.

1. Twenty-four-hour urinary excretions of nicotinic acid and amide, quinolinic acid, N'-methylnicotinamide, 6-pyridone, tryptophan, kynurenin, anthranilic acid and 3-hydroxyanthranilic acid were determined in 10 adult men before and after the feeding of 5 g. DL-tryptophan, on two consecutive days.

2. Normally, urine contained nicotinic acid and amide, quinolinic acid, N'-methylnicotinamide, 6-pyridone and tryptophan and excretions of these metabolites were increased after the feeding of tryptophan. The increase in the excretion of 6-pyridone was maximum.

3. Kynurenin and 3-hydroxyanthranilic acid which were absent in urine appeared after the feeding of tryptophan. Anthranilic acid could not be estimated in the urine even after the administration of tryptophan.

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STUDIES ON PROTEIN AND CARBOHYDRATE METABOLISM AS AFFECTED BY VITAMIN B₁₂ AND FOLIC-ACID DEFICIENCY IN RATS.

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ATTENTION has recently been drawn on the problem relating to inter-relationship between metabolism of proteins and vitamins and of carbohydrates and vitamins.

Hsu and Combs (1952) reported that in vitamin B₁₂-deficient chicks, there was increased blood levels of non-protein N, urea N, amino acid N, creatinine and uric acid. Zucker and Zucker (1948) also observed increased blood non-protein N in vitamin B₁₂-deficient rats. While Sahasrabudhe and Rao (1951) reported enhanced synthesis of liver protein and liver nucleic acid after injection of vitamin B₁₂ in rats, Ling and Chow (1952) and Rasch *et al.* (1955) could not find any change in the protein content of the liver of vitamin B₁₂-deficient rats or after administration of the vitamin.

In folic-acid deficiency blood uric acid is high which is markedly decreased after administration of folic acid (Zumoff, 1953). Dinning and Day (1949) reported that folic acid was in some way involved in creatine formation through its action on glycine metabolism.

Ling and Chow (1954) observed hypoglycemia and diminished glucose tolerance in vitamin B₁₂-deficient rats. They also observed (1953) decreased sulphhydryl groups in blood of vitamin B₁₂-deficient rats. Williams *et al.* (1949) reported that there is an inverse relation between folic-acid content of the diet and the blood glucose. Bruemmer *et al.* (1955) observed that glycogen content of liver of vitamin B₁₂-deficient rats was greatly diminished.

The above résumé indicated inter-relationship of protein and carbohydrate metabolism with vitamin B₁₂ and folic acid nutrition. The present investigation

was undertaken to study the protein and carbohydrate metabolism as influenced by the combined deficiency of vitamin B₁₂ and folic acid.

EXPERIMENTAL.

Weanling male rats weighing between 30 g. and 35 g. were fed a diet free from folic acid and vitamin B₁₂ (Fatterpaker *et al.*, 1955). The diet contained iodinated casein at 3 per cent level and 1 per cent of sulfasuxidine. It took about 5 weeks for the development of the combined deficiency of vitamin B₁₂ and folic acid. The normal control animals received in addition to the folic acid and vitamin B₁₂-deficient diet, a daily oral supplement of 0.5γ vitamin B₁₂ and 8γ folic acid per animal. A high mortality rate was noticed in the deficient animals. A group of 5 rats was housed in one metabolism cage and their 24-hour output of urine was collected in flasks containing 0.5 c.c. concentrated hydrochloric acid and 1 c.c. toluene. The different nitrogenous constituents of urine were determined in the normally fed animals and in animals during the progress of combined vitamin deficiency.

The deficiency in these vitamins was established by erythrocyte count and determination of hæmoglobin (Palmer, 1918). Deficiency of folic acid was further established by the microbiological assay of the folic acid and citrovorum factor contents of the liver (Flynn *et al.*, 1951; Sauberlich and Baumann, 1948; Sauberlich, 1949).

Twentyfour-hour urinary excretion of nitrogenous end-products in normal and deficient rats.—Total non-protein N was estimated by the determination of the nitrogen in an aliquot of protein-free filtrate by the micro-Kjeldahl method.

Urea N was estimated by treating an aliquot of urine with urease and determining the nitrogen by the micro-Kjeldahl method.

The amino acid N present in urine was estimated by the method of Pope and Stevenson (1939) as modified by Albanese and Irby (1944).

Creatinine and creatine in urine were estimated by the method of Folin (1914) as modified by Bonsnes and Taussky (1945) and Peters (1942).

Uric acid was estimated by the method of Benedict and Franke (1922).

Estimation of total protein, non-protein N and glucose contents of blood, and glycogen and total protein contents of liver and glycogen content of the muscles of normal and deficient rats.—Blood was collected by cardiac puncture in tubes containing oxalate. The normal and deficient animals were killed by decapitation after they were fasted for 18 hours. Livers were removed, a portion weighed immediately, dropped in a tube containing heated 30 per cent potassium hydroxide for digestion and final precipitation of the glycogen with alcohol. Another aliquot of liver was weighed, and a 20 per cent homogenate was prepared in 0.1 M acetate buffer of pH 4.5. This homogenate was utilized for the determination of folic acid and citrovorum factor, total protein and moisture content.

Total non-protein N of the blood was determined in protein-free blood filtrate by the micro-Kjeldahl method. Total protein of blood was determined by the

determination of nitrogen by the micro-Kjeldahl method. The nitrogen equivalent of non-protein N of the blood was deducted from the total nitrogen value and the nitrogen value was multiplied by 6.25. Liver protein was determined in the liver homogenate by the micro-Kjeldahl method. Blood sugar was determined by the method of Hagedorn and Jensen (1923). Liver and muscle glycogen were precipitated by the method of Grattan and Jensen (1940), precipitate was hydrolyzed and reducing sugar was estimated by the method of Hagedorn and Jensen (*loc. cit.*).

Studies on glucose tolerance test in normal and deficient rats.—As most of the deficient rats died after the fasting sample of blood was collected from the heart, it was not possible to perform the customary glucose-tolerance test in those animals. Therefore, the sugar was determined in the blood collected 2½ hours after the feeding of glucose (2 g. per kilo body-weight in a 60 per cent solution) in both the normal and deficient animals. It was observed that with the dose of glucose fed, it took 2½ hours in normal animals for the blood sugar to reach the normal level. The results are given in Tables I to V.

TABLE I.
Erythrocytes and hemoglobin contents of blood, and folic acid and citrovorum factor contents of liver of normal and combined vitamin B₁₂ and folic acid-deficient rats.

ERYTHROCYTES PER C.MM., MILLION:		HÆMOGLOBIN, g. PER CENT.		FOLIC ACID, r/g. DRY LIVER.		CITROVORUM FACTOR, r/g. DRY LIVER.	
Normal (22).	Deficient (22).	Normal (22).	Deficient (22).	Normal (11).	Deficient (15).	Normal (11).	Deficient (15).
*6.12±0.28 † 8.84	3.31±0.40	9.4±0.70 4.98	5.9±0.07	161.0±20.32 7.9	0.60±0.28	46.6±10.4 4.4	620.33±0.15

*Mean±S.E. †Figures in parenthesis indicate the number of animals.

DISCUSSION.

The diminution of the number of red blood cells and decrease in the hæmoglobin value in the animals fed a diet deficient in vitamin B₁₂ and folic acid indicated that the animals were deficient in both the vitamins. The hæmoglobin values reported for the normal animals, however, were low. This might be due to the method of estimation of hæmoglobin used in the present investigation which seems to be superior to the Sahli's method commonly used. The diminution in the folic acid and citrovorum factor values of the liver of the deficient animals indicated folic-acid deficiency. As the tissue contents of vitamin B₁₂ were not determined, it was not possible to know the degree of deficiency of vitamin B₁₂ in these animals but the absence of vitamin B₁₂ in the diet and the hematological picture warranted the conclusion that the animals were also deficient in vitamin B₁₂.

With the progress of combined deficiency of vitamin B₁₂ and folic acid, all the rats excreted increased amounts of the different nitrogenous constituents in urine with the exception of creatine. Persistent increased urinary excretions of non-protein N, urea N, ammonia N, amino acid N creatinine and uric acid in

TABLE II.

Twenty-four-hours urinary excretions of nitrogenous end-products of protein metabolism by rats during the progress of combined deficiency of vitamin B₁₂ and folic acid (4 groups of rats, each group containing 5 rats, were taken).

(Figures are expressed in mg. per 100 g. body-weight per 24 hours.)

			DAYS AFTER PLACING THE RATS ON THE DEFICIENT DIETS:				
			7	14	21	28	35
Non-protein nitrogen	Normal ..	..	*70.1±2.6	68.6±3.1	71.4±13.7	73.2± 2.8	72.0
	Deficient ..	..	107.6±9.4	119.2±9.5	173.2±26.3	252.0±19.4	360.2
	t ..	..	3.83	5.05	2.17	9.12	
Urea nitrogen	Normal ..	..	37.2±2.4	39.0±2.7	39.0± 1.4	40.4± 2.4	41.2
	Deficient ..	..	46.4±4.5	61.0±1.7	105.1±12.4	172.8±20.6	241.8
	t ..	..	1.81	6.89	5.31	6.37	
Ammonia nitrogen	Normal ..	..	19.6±2.5	19.2±2.3	20.2± 1.9	22.1± 2.1	22.5
	Deficient ..	..	26.3±2.7	26.5±3.9	36.4± 2.6	50.8± 2.5	92.6
	t ..	..	1.83	1.61	5.09	8.72	
Amino acid nitrogen	Normal ..	..	1.3±0.0	1.5±0.0	1.0± 0.0	1.0± 0.0	0.9
	Deficient ..	..	1.6±0.5	2.5±0.0	2.7± 0.2	3.8± 0.8	5.3
	t ..	..	1.07	1.0	7.45	3.33	
Creatinine	Normal ..	..	1.4±0.0	1.4±0.1	1.4± 0.1	1.6± 0.2	1.7
	Deficient ..	..	1.4±0.1	1.8±0.0	1.9± 0.0	2.6± 0.4	5.7
	t ..	..	0	3.63	7.14	4.0	
Creatine	Normal ..	..	0.4±0.2	0.8±0.0	0.5± 0.0	0.6± 0.0	0.7
	Deficient ..	..	0.4±0.1	0.8±0.0	0.5± 0.0	0.6± 0.1	0.7
	t ..	..	0	0	0	0	
Uric acid	Normal ..	..	0.4±0.2	0.5±0.0	0.4± 0.1	0.4± 0.1	0.5
	Deficient ..	..	0.5±0.1	0.7±0.0	0.9± 0.0	1.0± 0.1	2.0
	t ..	..	0.43	0.2	7.14	5.66	

* Mean±S.E.

TABLE III.

Protein content of liver and protein and non-protein N levels of blood in normal and vitamin B₁₂ and folic acid-deficient rats.

LIVER PROTEIN, G./100 G. DRY LIVER:		BLOOD PROTEIN, G./100 C.C. BLOOD:		BLOOD NON-PROTEIN N, MG./ 100 C.C. BLOOD:	
Normal (16).	Deficient (16).	Normal (16).	Deficient (16).	Normal (11).	Deficient (11).
*90.49±1.80 t 1.27	93.55±1.60	23.83±1.25 1.89	26.59±0.75	30.62±0.28 5.76	39.11±1.44

* Mean±S.E.

Figures in parenthesis indicate the number of animals.

TABLE IV.

Blood sugar values of rats before and 2½ hours after the feeding of glucose in normal and vitamin B₁₂ and folic acid-deficient rats:

Blood glucose (mg. per 100 c.c. blood.)

BEFORE THE FEEDING OF GLUCOSE:		TWO AND HALF HOURS AFTER THE FEEDING OF GLUCOSE:	
Normal (a) (18).	Deficient (b) (10).	Normal (c) (10).	Deficient (d) (10).
*79.1±1.72	26.8±1.94	77.3±2.28	87.2±5.60
t value between (a) and (b)		..	20.17
t " " (c) and (d)		..	1.64
t " " (a) and (c)		..	0.63
t " " (b) and (d)		..	10.20

*Mean±S.E.

Figures in parenthesis indicate the number of animals.

TABLE V.

Glycogen content of liver and muscle of normal and vitamin B₁₂ and folic acid deficient rats.

Glycogen (g./100 g. liver.)

GLYCOGEN CONTENT OF LIVER OF RATS NOT FED GLUCOSE:		GLYCOGEN CONTENT OF LIVER OF RATS, KILLED ½ HOURS AFTER FEEDING OF GLUCOSE:		GLYCOGEN CONTENT OF MUSCLE OF RATS NOT FED GLUCOSE:		GLYCOGEN CONTENT OF MUSCLE OF RATS, KILLED 2½ HOURS AFTER THE FEEDING OF GLUCOSE:	
Normal (a) (18).	Deficient (b) (10).	Normal (c) (10).	Deficient (d) (14).	Normal (a) (21).	Deficient (b) (10).	Normal (c) (10).	Deficient (d) (14).
*0.482±0.14	0.061±0.02	0.601±0.25	0.053±0.01	0.098±0.10	0.155±0.02	0.128±0.03	0.119±0.02
t value between (a) and (b)		..	2.90	t value between (a) and (b)		..	0.57
t " " (c) and (d)		..	2.20	t " " (c) and (d)		..	0.27
t " " (a) and (c)		..	0.41	t " " (a) and (c)		..	0.30
t " " (b) and (d)		..	0.34	t " " (b) and (d)		..	1.20

*Mean±S.E.

Figures in parenthesis indicate the number of animals.

vitamin B₁₂ and folic acid-deficient rats suggest that these vitamins are concerned in protein metabolism. No change in the protein values of liver and blood was observed in vitamin-deficient rats. This is in support of the observations of Ling and Chow (1952), Rasch *et al.* (*loc. cit.*) who did not observe any change in protein content of liver of vitamin B₁₂-deficient rats. The blood non-protein N increased significantly in combined vitamin B₁₂ and folic acid-deficient rats, which is in confirmation with the observations of Hsu and Combs (*loc. cit.*) and Zucker (*loc. cit.*).

A significant decrease in fasting blood sugar values was observed in vitamin B₁₂-folic acid-deficient rats. Even 2½ hours after the oral administration of glucose, the blood sugar levels in deficient animals were higher than the fasting levels, although in the normal animals the blood sugar value came down to normal level 2½ hours after the feeding of glucose. This indicated defective utilization of glucose in combined deficiency of vitamin B₁₂ and folic acid. Ling and Chow (1954) also observed hypoglycemia and diminished glucose tolerance in vitamin B₁₂-deficient rats. The glycogen value of the liver of the combined vitamin B₁₂ and folic acid-deficient rats decreased significantly. This value could not increase after the administration of glucose suggesting thereby, that these two vitamins play some part in the deposition of glycogen in the liver. The low fasting blood sugar of the deficient animals, therefore, is not due to the defect in the liver glycogenolysis. The muscle glycogen content is not altered in deficient animals, but the deposition of glycogen in the muscle of deficient animals did not increase after the administration of glucose. The low glycogen content of the muscle of normal animals is likely to be due to the fasting of the animals for 18 hours. A decrease in reduced glutathione and ergothioneine was observed by Register (1954), and Ling and Chow (1953) in vitamin B₁₂-deficiency. This, in turn, may affect the normal synthesis of insulin as insulin molecule contains cystine, an important source of which is glutathione, leading to diminished secretion of insulin and consequent defective utilization of glucose.

SUMMARY.

1. Combined deficiency of vitamin B₁₂ and folic acid was produced in weanling rats by feeding a diet deficient in above vitamins and containing iodinated casein and sulfasuxidine.

2. The urinary excretions of different nitrogenous constituents, such as non-protein N, urea N, uric acid, amino acid N, creatinine and creatine, were estimated during the progress of the combined vitamin deficiencies. All the nitrogenous constituents except creatine were excreted in increased amounts in vitamins-deficient animals.

3. Glucose, total protein and non-protein N content of blood and total protein contents of liver were determined in normal and deficient animals. No change in protein content of blood and liver could be obtained in the deficient animals. The non-protein N of blood was high and the fasting blood sugar level was low in the deficient animals.

4. Glucose content of the blood 2½ hours after the oral administration of glucose was determined in the normal and deficient animals. While, in normal animals the blood sugar value was similar to the fasting blood sugar value, the same in deficient animals was significantly higher.

5. Glycogen content of liver and muscle was determined both before and 2½ hours after the oral administration of glucose in both the normal and deficient animals. The deficient animals showed a very low glycogen content in the liver which did not increase after the administration of glucose. The muscle glycogen

of the deficient animals was unaffected and did not change after the administration of glucose.

6. Implications of these observations have been discussed.

Leuconstoc citrovorum culture was kindly supplied by Lederle Laboratories (India) Ltd. Financial assistance of Indian Council of Medical Research is gratefully acknowledged. One of the authors (M.M.) received the Kalyan Kumar Mukherjee Research Scholarship of the Calcutta University.

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THE INFLUENCE OF SOME AUTONOMIC DRUGS ON THE ACTIVITY OF THE SPONTANEOUSLY BEATING ISOLATED FROG'S HEART AND ITS SINO-AURICULAR CHAMBER.

BY

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INTRODUCTION.

THE most plausible hypothesis for the initiation and maintenance of cardiac rhythm is the operation of acetylcholine synthesis destruction cycle (Bulbring and Burn, 1949; Spadolini and Giachetti, 1956 (quoted by Burn, 1956). Experimental evidence based on the presence of acetylcholine (Burn, 1953-54), choline acetylase (Comline, 1946) and cholinesterase (Briscoe, 1954), and also the evidence of release of acetylcholine in the perfusate (Briscoe and Burn, 1954) and in the heart lung preparation of dog (Burn, 1954) along with the demonstration of excitatory effect of acetylcholine (Sachs, 1937; Rothberger and Sachs, 1938; Welsh, 1938; Spadolini and Domini, 1956 (quoted by Burn, 1956); Hoffmann *et al.*, 1954; McDowall, 1954; Burn and Vane, 1949; Briscoe and Burn, 1954), its depolarizing property (Sampson Wright, 1952), its ability to cause localized pacemaker potentials develop into conducted action potentials (Marshall and Vaughan Williams, 1954) and its relationship to experimental arrhythmias (Burn, Vaughan Williams and Walker, 1955) has considerably substantiated the acetylcholine hypothesis. Evidence has also been advanced in support of the hypothesis on the basis of the effect of anti-cholinesterase drugs.

During the course of investigating the mechanism of action of a drug on isolated frog's heart, certain important observations were made in connection with the effect of some autonomic drugs. As these observations were rather unexpected, interest was aroused to undertake a detailed study. The results so obtained serve as additional evidence in favour of the operation of acetylcholine cycle to maintain cardiac rhythm and also give additional facts which have to be explained by any

other hypothesis which may be proposed later in this connection. This work is incorporated in the present paper.

MATERIAL AND METHODS.

The frog's heart was considered to be the most suitable one for this purpose as the natural pacemaker in the sinus is easily accessible and the main ganglionic station (the crescent) is clearly visible as a gray line. The experiments were, therefore, conducted on isolated frog's whole heart and the isolated sino-auricular chambers. The heart was perfused with oxygenated Ringer at a pH 7.2 to 7.6, after introducing a Syme's perfusion cannula through the inferior vena cava and tying the ligature over the cannula. To obtain the isolated sino-auricular chamber the auriculoventricular groove was tied and the ventricle was removed. The duplication of observations on the isolated sino-auricular chamber was useful for analysing the influence of the drug on the auricular and the ventricular responses. The perfusion pressure was maintained strictly at a constant level of 2 cm. Ringer by using a overflow device in order to obviate changes in the force and frequency of heart beat due to fluctuations in the perfusion pressure (Pathak, 1958). To study the influence of the prolonged contact of various concentrations of the drugs, the isolated frog hearts were suspended in oxygenated Ringer solution in a 100 c.c. bath. The mechanical records were taken on a slowly moving smoked kymograph paper. Each drug was studied on 15 hearts and in total 60 hearts were used. The drug was not repeated more than thrice on any heart and fresh trial was only made when the heart had completely recovered from the effects of previous dose as evidenced by the return of the kymographic record to normal. The following drugs were studied:—

1. Nicotine.
2. Homatropine and atropine.
3. Eserine.
4. Adrenaline.

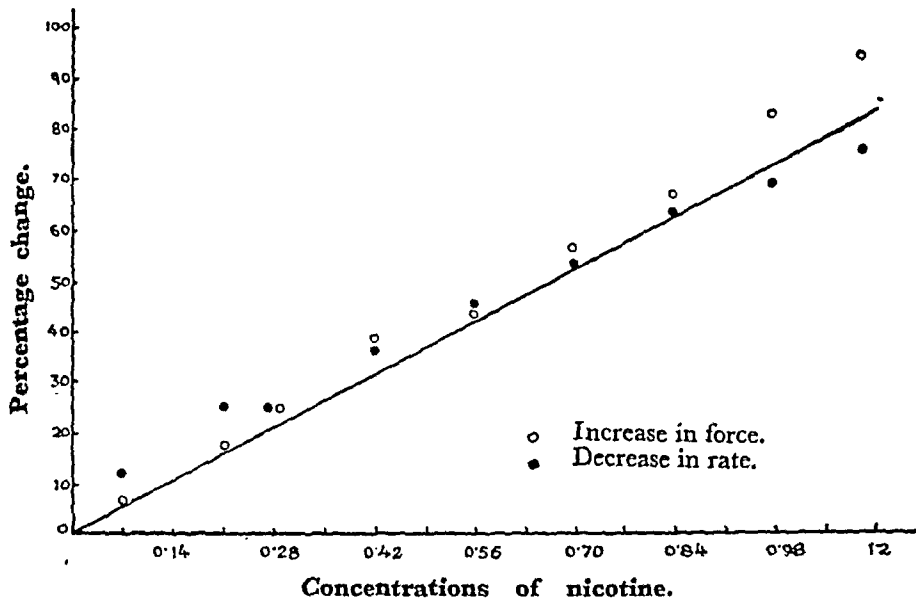
A wide range of concentration of each drugs was studied. The drugs were sometimes used in rather higher doses than conventional in order to obtain some of these results, as in lower doses some of the effects were either insignificant or were completely obscured. The intention during the present work was not to duplicate the known pharmacological actions but to find out a deviation from them. Hence, the use of higher than conventional dose was absolutely justified.

RESULTS.

The action of each drug is considered separately in details below:—

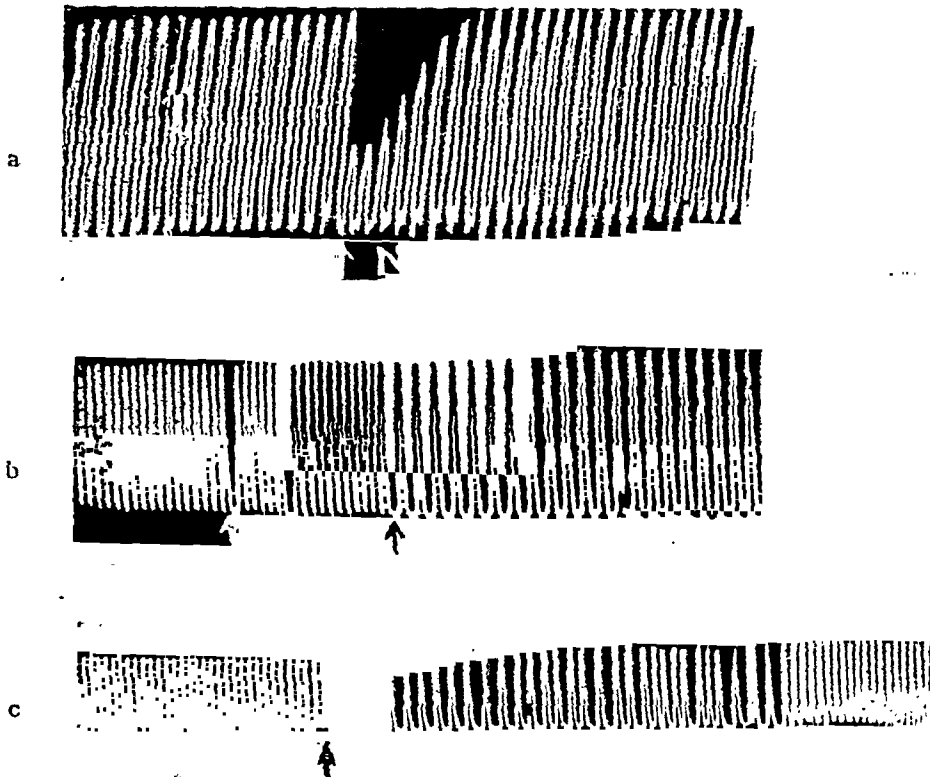
Nicotine.—In lower concentrations nicotine caused a progressive decrease in the rate with a simultaneous increase in the amplitude of contraction (Text-figure 1b) in a linear fashion (the Graph). This differential effect on the inotropic and chronotropic responses was obtained in concentrations ranging from 0.07 per cent to 1.2 per cent or more. These results are given in the Table and the Graph illustrates the relation of various concentrations to percentage change in the force and frequency of heart beat. Higher concentrations gave an inhibitory effect on both the inotropic and chronotropic responses. The negative inotropic

GRAPH.



Differential effect of nicotine on the percentage change in the inotropic and chronotropic responses of the frog's isolated heart.

TEXT-FIGURE 1.



(a) *Inhibitory effect of nicotine.* (b) *Differential effect of nicotine on the chronotropic and inotropic responses.*
(c) *Stoppage of heart beat by nicotine.*

and the negative chronotropic effects increased in intensity as the concentration or the total dose of the drug was increased. At higher concentrations the inhibitory effect on the inotropic response was quite prompt (Text-figure 1a). The inhibitory effect could produce a complete stoppage of the heart beat (Text-figure 1c). External application of 2 to 4 per cent solution of nicotine over the crescent gave an inhibitory effect in 90 per cent of the trials and a negative chronotropic effect associated with a positive inotropic effect in the remaining 10 per cent of the trials. Intraluminal administration of the drug was more effective. An excitatory effect was also obtained in some trials.

TABLE.

Differential action of nicotine on the inotropic and chronotropic response.

Concentration, per cent.	Percentage decrease in frequency.	Percentage increase in force.
0.07	12.5	5.5
0.21	25.0	16.5
0.28	25.0	25.0
0.42	37.5	38.8
0.56	43.7	44.4
0.70	56.2	52.7
0.84	62.5	66.6
0.98	68.7	83.3
1.20	75.0	94.4

Homatropine or atropine.—In lower concentrations ranging from 0.01 per cent to 0.05 per cent or more homatropine caused a progressive decrease in both the rate and amplitude of contraction. Higher concentrations were strongly inhibitory (Text-figure 2a), specially when administered intraluminally and stopped the heart beat. External application of 1 per cent solution of homatropine on the crescent resulted in a decrease both in the rate and amplitude of contraction. Dissociation of action on the rate and amplitude was possible, e.g., the amplitude could increase without any significant variation in the rate (Text-figure 2b). Homatropine did not cause a simultaneous increase both in the force and frequency of beat.

Eserine.—The effect of eserine in lower concentrations was similar to that of nicotine. Concentrations of eserine ranging from 0.01 per cent to 0.05 per cent or more showed a progressive decrease in the heart rate with a simultaneous increase in the force of contraction. This effect was obtained in 85 per cent of the trials. In 10 per cent of the trials an excitatory effect more marked on the inotropic response was obtained. In the remaining 5 per cent of the trials, however, no immediate effect was noted on the inotropic or chronotropic responses even on repetition. This was true for some hearts even when the drug was administered intraluminally in as high concentration as 0.5 per cent. In the eserine responsive hearts higher concentrations, when used intraluminally, were strongly inhibitory (Text-figure 3a) and proved effective in arresting the heart beat (Text-figure 3b). The baseline was raised. Administration of homatropine or atropine at this point

is, however, a clear indication that the action of these drugs may not be 'direct' in the real sense and may be conditioned by certain intrinsic mechanisms of the heart.

In view of the hypothesis of the operation of acetylcholine synthesis-destruction cycle for the initiation and maintenance of heart beat, these results assume a particular importance. It has been proposed that acetylcholine is synthesized at the pacemaker with the help of choline acetylase and when a critical level of acetylcholine concentration is reached a heart beat follows. The released acetylcholine is quickly destroyed with the help of another enzyme cholinesterase. Thus, a cycle of acetylcholine synthesis and destruction is supposed to operate to maintain cardiac rhythm. Acetylcholine is a well-known depolarizing agent and a correlation of acetylcholine synthesis and release with the observations on the electrical activity at the pacemaker (Marshall and Vaughan Williams, *loc. cit.*) is possible. Electrical studies indicate that at the pacemaker, first a small localized potential is developed, which on attaining a critical height gives rise to a conducted response. It is, therefore, possible that the released acetylcholine acts as depolarizing agent to set up the localized small pacemaker potential which reaches a critical height at a certain concentration of acetylcholine and leads to a conducted response. The effects of the drugs studied are discussed below with the above background in view.

Effect of nicotine.—The heart was always found to be responsive to nicotine. External application of nicotine over the crescent gave either a pure inhibitory effect or a negative chronotropic effect associated with a positive inotropic effect. Nicotine is known to stimulate the autonomic ganglion cells in small doses and paralyse them in higher concentrations. In the case of the isolated frog's heart the parasympathetic ganglion cells are present at the crescent and give rise to the post ganglionic vagal fibres. The application of nicotine over the crescent will cause either stimulation or paralysis of these neurones. The stimulating effect can be expected to lead to inhibition of the heart but the paralysis of these neurones cannot be expected to cause any effect on the spontaneously beating isolated heart. Thus, the inhibitory effect of nicotine can be explained on the basis of stimulation of the parasympathetic neurones present in the crescent although the idea is not very convincing on closer analysis. The occasional excitatory effect of nicotine is difficult to explain specially in view of the fact that presence of sympathetic neurones has never been reported in the crescent. Even if it is argued that sympathetic neurones might be present at the crescent, one would have to further assume that they overbalance the parasympathetic neurones or one would have to postulate a differential sensitivity to the action of nicotine in the concentrations used before the excitatory effect of nicotine can be explained on the basis of its known pharmacological actions. It is, however, possible that the heart which showed the excitatory effect of nicotine was working under suboptimal conditions and that the acetylcholine synthesis was below normal. Under such circumstances the additional quantity of acetylcholine released as a result of parasympathetic neurone stimulation at the crescent restored optimal concentration of acetylcholine necessary for excitatory effect and thus increased the activity of the heart. Another possibility regarding the excitatory effect of nicotine can be the possible release of epinephrine-like substance due to the action of acetylcholine

released as a result of crescentic stimulation. Such a phenomenon has been described by Hoffmann *et al.* (*loc. cit.*) in atropinized hearts. It is, however, possible that nicotine applied externally over the crescent spread to the other parts and the results obtained are due to a direct action of the drug on the heart. The possibility of direct or indirect action of nicotine on the acetylcholine synthesis-destruction cycle, however, cannot be ruled out. The slowing and arrest of the beat support this possibility.

Effect of homatropine and atropine.—Homatropine caused a decrease in the rate with a simultaneous decrease in the amplitude of contraction or the amplitude increased without any noticeable effect on rate. Homatropine is known to block the action of acetylcholine presumably because of a substrate competition for the same receptors. A decrease in the rate leading to stoppage of the heart could be explained by complete blockage of the action of endogenously produced acetylcholine. The slowing and stoppage of heart cannot be explained otherwise. Although atropine or homatropine blocks the action of acetylcholine, it does not check the formation and release of acetylcholine. Hence, the acetylcholine continues to accumulate. It is possible that on reaching a critical concentration the blocking action of atropine is more than overcome and greater depolarization follows which increases the amplitude of contraction. Another possibility can be that the particular heart in which excitatory effect of homatropine was observed was already producing acetylcholine in so large quantities due to its high rate of synthesis that normally it was exerting some inhibitory effect. If homatropine is administered the inhibitory influence of acetylcholine may be blocked and its excitatory effect may be enforced.

The proposition that endogenously produced acetylcholine becomes sensitive to atropine only after release in the blood or perfusion fluid (Burn, 1956) is not supported by the evidence collected in the present series of experiments; on the contrary, homatropine even on external application on the crescent resulted in a prompt slowing of the heart even to the point of complete stoppage. Thus, homatropine did influence the spontaneous activity of the heart, which is supposed to be maintained by the endogenously produced acetylcholine. The mechanism of action of homatropine in causing the slowing or stoppage of the heart may be through its blocking action on acetylcholine at the pacemaker or in the conducting tissue but the point is that it did block the action of endogenously produced acetylcholine. Probably, the difference of results of the action of atropine between the experiments of Burn (1956) and the present experiments is due to larger dose of atropine than that used by Burn.

Effects of eserine.—Eserine administration promptly caused a decrease both in the rate and amplitude of contraction in 85 per cent of the trials and an excitatory effect more pronounced on the amplitude in 10 per cent of the trials. In the remaining 5 per cent of the trials no effect was observed on the chronotropic or inotropic response. Eserine is a well-known anti-cholinesterase drug. Inhibition of cholinesterase by eserine will result in a decrease or arrest of hydrolysis of acetylcholine which will, therefore, accumulate and exert its inhibitory effect. Thus, the decrease in amplitude and rate can be easily explained on the basis of anti-cholinesterase property of eserine. The excitatory effect of eserine are difficult

to understand unless one presumes that the hearts were working under suboptimal conditions and the choline acetylase system was not fully active. Accumulation of acetylcholine by the addition of eserine reactivated the choline acetylase system and augmented the beat of the heart. Another possible explanation is the release of epinephrine-like substance. The most interesting finding which defies all such explanations is the unresponsiveness of some hearts to the action of eserine even in quite high doses and the observation that a heart responsive to the action of eserine at one time may be completely unresponsive in the next trial.

Effect of adrenaline.—In the case of adrenaline, also, some hearts were either not responsive at all or became unresponsive in some trials or the other. Those which were responsive to adrenaline showed both excitatory and inhibitory effects. It is a common belief that adrenaline stimulates the heart by raising the metabolic level of cardiac tissue. A raised metabolic level of the pacemaker will lead to an increased synthesis of acetylcholine and may cause greater depolarization. If both the rate of synthesis and the quantity of acetylcholine synthesized are increased a quicker and stronger beat is possible specially when the cholinesterase system is also activated. The inhibitory effects of adrenaline also can be explained on the above presumption. In case the acetylcholine synthesized is very large in quantity and is not destroyed with an increased rate, it will accumulate and exert its inhibitory effects. It is, therefore, possible that adrenaline may be acting as a stimulant both for the choline acetylase and cholinesterase systems.

The explanations and arguments advanced above lead to the conclusion that acetylcholine reactive surfaces and acetylcholine synthesizing tissue may be involved in the action of these drugs. Thus, the site of actions of these drugs may be the same as that of acetylcholine. The acetylcholine receptors may not, therefore, be very specific or that acetylcholine synthesis-destruction cycle may be sensitive to the action of these drugs so that the rate of synthesis and the quantity of acetylcholine synthesized are readily altered. Acetylcholine in larger quantities itself exerts a negative catalytic action on its synthesis. The evidence collected in the present study strongly supports the possibility of operation of acetylcholine synthesis-destruction cycle for the initiation and maintenance of cardiac rhythm for otherwise the results are difficult to explain.

SUMMARY.

The actions of various concentrations of four autonomic drugs including adrenaline, nicotine, eserine and atropine have been studied on spontaneously beating isolated frog hearts. It has been found that all the four drugs give both excitatory and inhibitory effects. A pure excitatory effect leading to simultaneous acceleration and augmentation of the heart beat was only common with adrenaline. The excitatory effect of atropine, nicotine or eserine was only occasional and was more marked on the inotropic response. The inhibitory effect of all the drugs can be easily obtained specially at higher concentrations. Both the inotropic and chronotropic responses were inhibited and the heart beat could be completely arrested. Neither the excitatory nor the inhibitory effect was universal. Some hearts were either unresponsive from the beginning or became unresponsive in

some trial or the other to the action of eserine, atropine and adrenaline. The possible mechanism of action of these drugs has been discussed in the light of the proposed hypothesis of operation of acetylcholine synthesis destruction cycle for the initiation and maintenance of heart beat. These observations lend further support to such a view for otherwise the results are difficult to explain. It has been suggested that the site of action of these drugs may be the acetylcholine synthesising tissue or the acetylcholine reactive surfaces.

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EFFECT OF DIETARY PROTEIN, CALORIES AND PYRIDOXINE ON GLUTAMATE-OXALOACETATE TRANSAMINASE ACTIVITY OF BLOOD.

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INTRODUCTION.

THE function of vitamin B₆ in protein metabolism is partly explained by its rôle in amino acid decarboxylation and transamination. Pyridoxal phosphate has been shown to be a prosthetic group in the enzyme systems concerned with these reactions (Umbreit and Gunsalus, 1945; Lichstein, Gunsalus and Umbreit, 1945).

The activity of different transaminase in animal tissues has been found to depend on pyridoxine nutrition of the animal (Brin, Olson and Stare, 1954; Shwartzman and Hift, 1951; and Meister, Morris and Tice, 1953). It has been shown by Coldwell and McHenry (1953) that glutamate-oxaloacetate transaminase (GOT) of rat liver is more sensitive to pyridoxine deficiency than glutamate-pyruvate transaminase. Recently, Marsh, Greenburg and Rinhard (1955) successfully adopted the method of Tonhazy, White and Umbreit (1950) for the estimation of GOT in whole blood. These authors concluded that in both monkeys and man, increase in the intake of pyridoxine in the diet resulted in a significant increase in the blood levels of transaminase and vitamin B₆. Recent studies have added serum GOT to the list of clinically useful enzymes (La Due, Wroblewski and Karman, 1954; Steinberg and Ostrow, 1955).

Tulpulé and Williams (1955) have reported that on low-protein-vitamin B₆ deficient ration, the loss of vitamin B₆ in different organs of the rat was markedly less than in the adequate protein vitamin B₆ deficient controls. However, when an adequate protein diet was given to protein starved animals, vitamin B₆ levels in liver, kidneys and intestine were significantly reduced. These observations

support the view of Beaton and McHenry (1953) that under normal physiological conditions, increase in the demand for vitamin B₆ in diet is proportional to protein intake.

In view of these observations it is reasonable to assume that at a certain minimum level of pyridoxine intake, transaminase activity of blood and other organs would respond to varying levels of protein intake, since under certain conditions, i.e. on low protein diet, the apo-enzyme would be a limiting factor in determining transaminase activity.

In the present investigations the effect of low and normal protein diets with or without vitamin B₆ on blood GOT activity in albino rats has been studied. Since the state of protein malnutrition in human beings throughout the world is often complicated by the concurrent caloric deficiency, it was considered worthwhile determining the influence on blood transaminase of caloric restriction in combination with low protein. An attempt has also been made to investigate the ability of the animal body to restore blood transaminase activity after initial depletion on the types of diets discussed above.

EXPERIMENTAL.

One hundred and four young adult rats (64 males and 40 females) of about 6 to 8 weeks in age and reared on the stock colony diet were distributed into six groups, which included three types of dietary regimens, viz., adequate protein (groups 1 and 2), low protein (groups 3 and 4) and low protein, restricted calories (groups 5 and 6). One-half the number of animals on each regimen (groups 1, 3 and 5) received pyridoxine supplement in the diet, whereas the other half (groups 2, 4 and 6) did not. For technical convenience the experiment was conducted on three different batches of animals, care being taken to distribute littermates equally in different groups and also the initial weights and the ages of the rats being kept, as far as possible, varying within narrow limits. Details of the experimental diets are given below.

Group 1 received a ration (basal diet) containing 70 parts of sugar, 20 parts of vitamin-free casein, 5 parts of sesame oil, 4 parts of salt mixture (McCollum and Davis, 1918) and one part of vitamin mixture. The vitamin mixture provided per 100 g. ration, thiamine 200 µg., riboflavin 300 µg., niacin 1.5 mg., calcium pantothenate 2.0 mg., pyridoxine hydrochloride 150 µg., biotin 10 µg., folic acid 20 µg., i-inositol 10 mg., para-amino-benzoic acid 10 mg., vitamin B₁₂ 1.5 µg. and choline chloride 100 mg. One hundred I.U. of vitamin A and 15 I.U. of vitamin D in sesame oil were administered weekly to each rat.

Group 2 received the same diet as group 1 except that pyridoxine was omitted from the vitamin mixture.

In group 3, the protein level in the basal diet was reduced to 2 per cent and the difference was made up with sucrose. Group 4 received 2 per cent protein diet without pyridoxine.

Groups 5 and 6 were allowed only one-half of the quantity of ration consumed by the rats in groups 3 and 4, respectively. To ensure a comparable intake of protein in groups 3 to 6, the protein content of the diets of groups 5 and 6 was

increased to 4 per cent as against 2 per cent in groups 3 and 4. Group 5 was made comparable to group 3 with respect to pyridoxine supplement by raising the level of this vitamin in the diet of group 5 to 300 μ g. per cent. Group 6 did not receive any vitamin B₆ in the diet.

All the animals were weighed every week. Blood was drawn also every week by cardiac puncture from rats in each of the groups for the study of transaminase activity. 0.5 c.c. of blood was immediately taken in 1.0 c.c. distilled water and shaken vigorously to achieve complete hæmolysis. GOT activity of blood was estimated by the method of Tonhazy *et al.* (*loc. cit.*) with slight modification. Suitable blanks were run in each to allow for the preformed pyruvate and ketoglutarate in blood. It was observed that the enzyme was stable up to 24 hours, when blood samples were preserved at 0°C.

After four weeks, re-alimentation was started on half the number of animals in each group with a view to seeing the effect of repletion with the missing nutrient in each of the dietary regimens under investigation. The remaining half in each of the groups continued to receive the same diet as before. Rats in group 1 with adequate protein and pyridoxine in their diet served as a positive control group and their diet remained unchanged throughout the experimental period. The information regarding the composition of diets of the animals in different groups before and after re-alimentation is given in Table I. Re-alimentation period extended to 2 to 3 weeks after the initial 4 weeks period and blood transaminase studies were done as before at weekly intervals.

TABLE I.

Dietary regimens before and after re-alimentation.

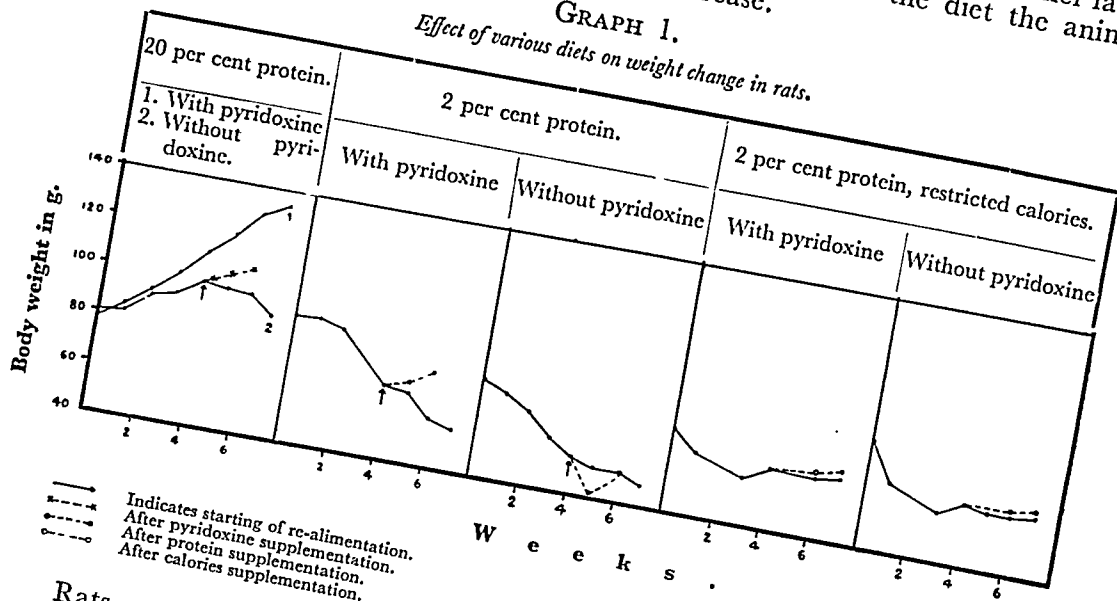
Group.	DIET AT START:				DIET ON RE-ALIMENTATION:			
	Number of rats.	Protein, per cent.	Pyridoxine HCl μ g./100g. diet.	Calories.	Number of rats.	Protein, per cent.	Pyridoxine HCl μ g./100g. diet.	Calories.
1	13	20	150	<i>Ad lib.</i>	12	20	150	<i>Ad lib.</i>
2	13	20	..	" "	2(a) 7	20	..	" "
					2(b) 5	20	150	" "
3	14	2	150	" "	3(a) 8	2	150	" "
					3(b) 6	20	150	" "
4	13	2	..	" "	4(a) 7	2	..	" "
					4(b) 6	20	..	" "
5	19	4	300	Restricted 50 per cent.	5(a) 8	4	300	Restricted 50 per cent.
					5(b) 8	2	150	<i>Ad lib.</i>
6	17	4	..	" " "	6(a) 8	4	..	Restricted 50 per cent.
					6(b) 8	2	..	<i>Ad lib.</i>

RESULTS AND DISCUSSION.

Growth.—The average weekly gain or loss in the body-weights of rats in various groups is presented in Graph 1. It can be seen that the growth rate of group 2 (20 per cent protein, — vitamin B₆) was comparable to that of group 1 (20 per cent protein, + vitamin B₆) for the first two weeks only to decrease markedly at the end of this period. From 4th to 7th week these rats showed a further fall in body-weight. However, when pyridoxine was restored in the diet the animals receiving the supplement showed a steady increase.

GRAPH 1.

Effect of various diets on weight change in rats.



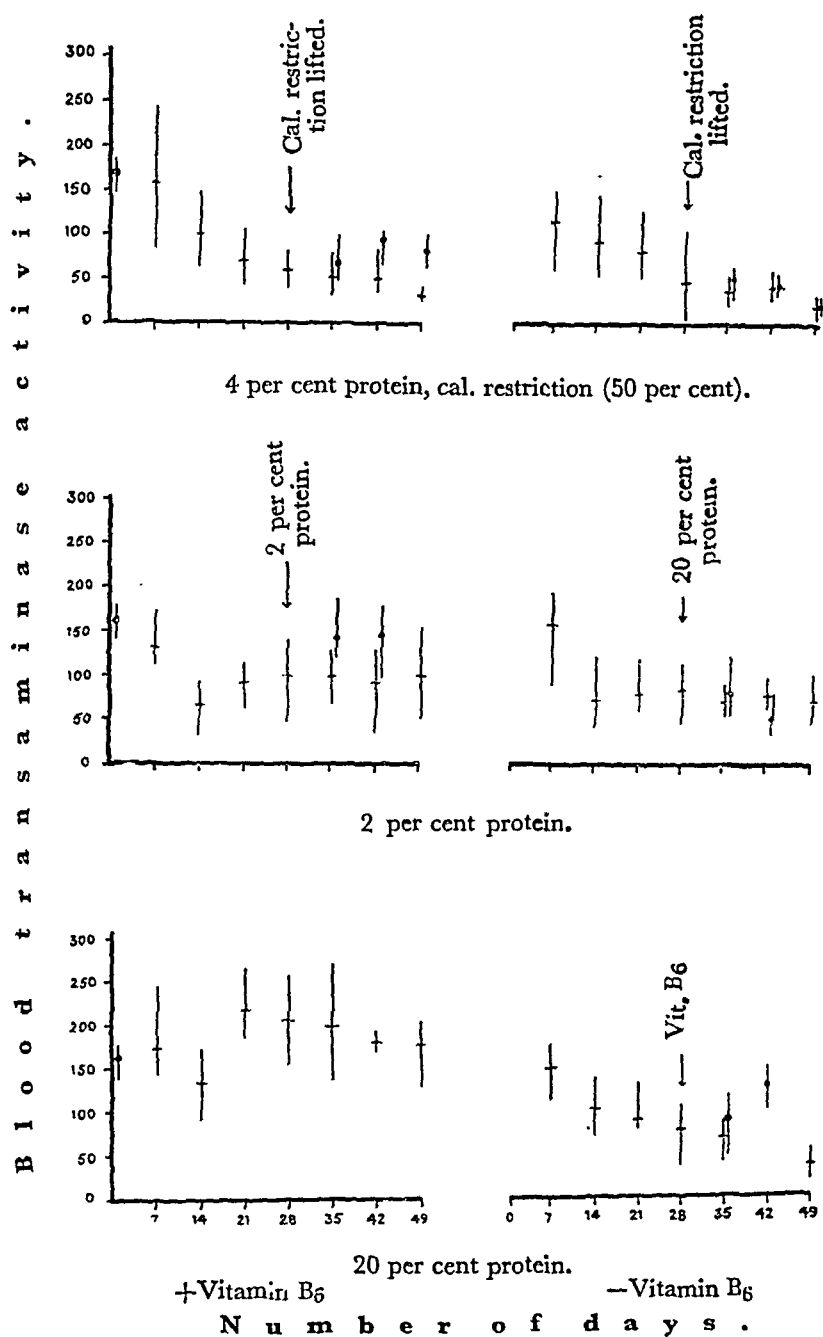
Rats on low protein diets (groups 3 and 4) lost weight at the rate of nearly 6 g. to 8 g. per week for the first four weeks; later the loss in body-weight was not appreciable. The initial loss of body-weight was uniform in both, pyridoxine supplemented (group 3) and pyridoxine deficient (group 4) animals. When normal protein was restored in the diet, pyridoxine-supplemented group slowly gained weight but pyridoxine-depleted group showed a marked reduction as would be expected from the increased demand for pyridoxine with the increased level of protein in the diet (Beaton and McHenry, *loc. cit.*). It was interesting to note, however, that these rats put on some weight during the last week of the experiment.

Semi-starved animals (groups 5 and 6) lost weight appreciably during the first two weeks and maintained it at the lower level thereafter over the remainder of the experimental period. This initial loss in body-weight was more in group 6, where vitamin B₆ deficiency was superimposed on low protein and restricted calories. Restoration of calories in the diet in the second phase of the experiment did not appreciably affect the weights of these rats.

Effect of Protein on Transaminase Activity of Blood.

Blood transaminase activity.—The results of blood transaminase activity are given in Table II, and the average values of various groups are represented in Graph 2.

GRAPH 2.



† Value for stock rats.
 † Without supplementation.
 † With supplementation.

Effect of different diets on blood GOT activity of rat.

TABLE II.
Blood glutamate-oxaloacetate transaminase activity of experimental rats.

Group number.	Diet.	BLOOD GLUTAMATE-OXALOACETATE TRANSAMINASE **													
		7th day.		14th day.		21st day.		28th day.		35th day.		42nd day.		49th day.	
		Number of obs.	Average.	Number of obs.	Average.	Number of obs.	Average.	Number of obs.	Average.	Number of obs.	Average.	Number of obs.	Average.	Number of obs.	Average.
1	Twenty per cent protein + vitamin B ₆ ..	10	173 ± 21.7†	14	135 ± 7.1	10	219 ± 7.9	13	203 ± 7.5	12	197 ± 12.3	4	178 ± 5.5	7	175 ± 9.9
2	(a) Twenty per cent protein — vitamin B ₆ ..	10	152 ± 5.8	16	92 ± 5.3	8	88 ± 8.2	13	72 ± 5.6	7	63 ± 5.9	..	..	7	32 ± 5.2
	(b) After vitamin B ₆ supplementation ..	..	..	..	..	..	..	..	..	4	84 ± 16.9	5	126 ± 8.7	..	..
3	(a) Two per cent protein + vitamin B ₆ ..	10	127 ± 6.6	15	63 ± 5.7	8	92 ± 8.1	14	100 ± 8.1	8	98 ± 7.6	5	92 ± 17.5	6	99 ± 17.7
	(b) After protein supplementation ..	..	..	..	..	..	..	..	..	6	146 ± 10.0	6	143 ± 14.3	..	..
4	(a) Two per cent protein — vitamin B ₆ ..	10	158 ± 10.0	16	68 ± 4.9	10	79 ± 7.1	13	83 ± 7.1	7	70 ± 4.3	5	81 ± 7.3	5	71 ± 10.0
	(b) After protein supplementation ..	..	..	..	..	..	..	..	..	6	84 ± 11.8	5	55 ± 9.1	..	..
5	(a) Four per cent protein + vitamin B ₆ caloric restriction (50 per cent) ..	20	149 ± 11.1	17	91 ± 5.5	18	63 ± 4.4	19	55 ± 3.1	8	46 ± 6.3	7	48 ± 5.9	4	29 ± 3.3
	(b) After caloric supplementation ..	..	..	..	..	..	..	..	..	8	60 ± 6.6	7	87 ± 5.0	6	79 ± 5.4
5	(a) Four per cent protein — vitamin B ₆ caloric restriction (50 per cent) ..	19	114 ± 5.7	17	91 ± 7.1	18	81 ± 6.0	17	47 ± 7.3	8	37 ± 4.7	8	43 ± 3.8	8	21 ± 3.2
	(b) After caloric supplementation ..	..	..	..	..	..	..	..	..	8	51 ± 5.5	7	43 ± 3.9	7	24 ± 2.4

* Transaminase unit— μ g. of pyruvic acid formed by one c.c. of blood during 10 minutes reaction at 37°C.

† Standard error of the mean.

Adequate protein diet with pyridoxine (group 1).—For the first two weeks of the experiment, the rats in this group (positive control group) showed transaminase activity of blood well within the normal range observed in the stock rats of comparable age (159 ± 20 units). At the end of the third week the enzyme activity in this group increased to 219 units and then it gradually declined to 175 units in the next five weeks. This variation, however, was statistically not significant. The protein content of the colony stock diet and the diet of group 1 being almost the same, the initial rise in blood transaminase activity could possibly be due to higher level of pyridoxine in the experimental diet. That this increase in the enzyme activity was not due to the increasing age of the animals has been confirmed by observations on stock rats at the same time intervals as the experimental rats. The former showed a range of 158 to 176 transaminase units per c.c. of blood.

Adequate protein diet without pyridoxine (group 2).—Blood transaminase activity in this group progressively fell as the experimental period progressed. On comparing these results with those obtained in group 1 the decrease in activity was found significant only after 28 days. At the end of the seventh week the enzyme activity in group 2 was reduced to about one-fifth of the initial value. When pyridoxine was supplemented after four weeks to some animals in this group, their blood transaminase activity showed a significant rise after 14 days, i.e., on the 42nd day of the experiment. For the significance test, the average value of enzyme activity in non-supplemented animals on 42nd day was extrapolated from the analytical data on 35th and 49th day of the experiment. This was possible because the fall in blood transaminase activity in nonsupplemented group was gradual throughout the experimental period.

Low protein diet with pyridoxine (group 3).—When rats were fed on 2 per cent protein diet supplemented with pyridoxine a sudden and significant reduction in blood transaminase activity was observed on 14th day followed by an appreciable rise in the enzyme activity on 21st day. This rise was significant when compared to the values on 14th day. However, when compared to the values at corresponding period in the control group (group 1) the enzyme activity on 21st day was significantly lower. After 21 days, the level of the enzyme in group 3 was maintained constant up to 7 weeks. This initial drop in the enzyme activity followed by the rise which was maintained later on suggested that there existed an adaptation mechanism which came into play after a certain period of feeding low protein diet. This adaptive mechanism, however, could not fully cope with the demand for protein for the maintenance of transaminase activity of the blood within the normal range.

When the level of protein was raised to 20 per cent, there was a significant rise in blood transaminase activity after one week which was maintained at the higher level throughout the period of supplementation.

It would be interesting to compare the results of group 2 (20 per cent protein, —vitamin B₆) and group 3 (2 per cent protein, +vitamin B₆). In group 2, transaminase activity of blood decreased significantly only after 28 days. When pyridoxine was supplemented to the diet at this stage, only a slight increase in the enzyme activity was observed after one week although it returned to normal at the end of the second week. On the other hand, when the low protein diet was fed with pyridoxine (group 3) the enzyme activity decreased to nearly 40 per cent

in two weeks and after restoration of normal protein in the diet the activity returned to normal within one week. These observations suggest that the apo-enzyme or the protein moiety of blood transaminase was dependent on dietary protein and responded rapidly to changes in the levels of its intake, whereas the changes in the co-enzyme due to ingested pyridoxine were more gradual.

Low protein diet, without pyridoxine (group 4).—On 2 per cent protein diet the initial drop in the enzyme activity was almost of the same magnitude in pyridoxine supplemented (group 3) and pyridoxine deficient (group 4) animals. In group 4 the enzyme activity was maintained at a reduced level up to 7 weeks for which period these experiments lasted, whereas in group 3, where the diet contained pyridoxine the activity was kept up at an elevated level after three weeks. However, there was no significant difference in blood transaminase activity measured between 14th and 49th days in these two groups indicating that the lowered enzyme activity was predominantly due to low protein in the diet.

In the second phase of the experiment, when protein alone was raised in the diet of group 4, there was no significant change in blood transaminase activity even after two weeks. Moreover, a slight but insignificant reduction in the enzyme activity was observed at this period. This was in contrast with the observations in group 3. Thus, it appeared that relatively small amounts of tissue pyridoxine in the rats fed on pyridoxine deficient diet (group 4) were gradually 'used up' when normal protein was restored in the diet and this was subsequently responsible for the reduced blood transaminase activity. These observations also conformed to the view that the blood transaminase activity was more sensitive to protein intake.

Restricted calorie, low protein diets with and without pyridoxine (groups 5 and 6).—When calorie restriction was superimposed on protein deficiency the blood transaminase activity showed a gradual and significant fall throughout the experimental period of 7 weeks. Restoration of adequate calorie intake in this group, keeping protein intake constant, raised the enzyme activity significantly after 14 days which was maintained at this level thereafter. However, this value was significantly lower than that of the positive control group. On the other hand, group 6, in which the diet was deficient in pyridoxine, in addition to protein and calories, the reduced level of blood transaminase was unaffected after restoration of only calories in the diet. This could probably be explained by the fact that when calories were restored in the diet more protein was made available for its specific rather than for energy purposes and when pyridoxine was present in the diet (group 5) it resulted in the increased transaminase activity. No such rise in enzyme activity could be observed in group 6 where the diet was deficient in pyridoxine.

It was observed by Kshirsagar and Patwardhan (unpublished) that in rats kept on 2 per cent protein diet fed *ad lib.* the liver transaminase activity showed a fall after 14 days. The superimposition of calorie restriction prevented this fall. This beneficial effect of calorie restriction progressively diminished and was lost between 42 and 56 days which suggested that the severity of changes due to inadequate protein was delayed when the calorie intake was restricted. On the other hand, transaminase activity in the intestine was not affected by protein depletion with or without calorie restriction. The present investigation seems to indicate that calorie restriction had no beneficial effect on blood transaminase

All these studies reveal the importance of the skin in lipid metabolism and suggest that this easily accessible portion of the human body may provide a new source of knowledge concerning the factors that affect cholesterol synthesis. The concentration of serum cholesterol has now been shown to be influenced by some dietary factors, specially dietary fats; and by certain disease states. The study of the skin under these conditions may be expected to provide a clue to the understanding of the factors underlying these changes. The present investigation is an attempt in this direction.

MATERIALS AND METHODS.

(a) *Composition of 'normal' sebum.*—Six healthy adult male volunteers between the age of 22 and 35 years were taken up for the investigation.

Sebum was collected by the procedure described by Mackenna *et al.* (*loc. cit.*). The arms were washed with soap and water and after thoroughly drying them, the subjects were instructed to avoid contact of the hands with any object. Exactly one hour after wash, the forearm was immersed up to the elbow in about 2.5 litres of distilled acetone kept in a tall glass cylinder. The arm was kept immersed for a period of 3 minutes; it was then removed and the other arm similarly extracted. Likewise, in the course of 24 hours, 3 sittings were given to each individual or six extractions made. Acetone was then removed by distillation, leaving a residue of the extracted fat, debris and dirt. The residue was dissolved in chloroform, the solution filtered and evaporated to dryness and the residue weighed. The sebum thus obtained was a yellow-brown plastic solid. The weighed amount of sebum was subsequently dissolved in 50 c.c. of chloroform and an aliquot was taken for the estimation of squalene and cholesterol.

Squalene was determined by the iodometric method described by Wheatley (1953). For the determination of cholesterol and its ester, the method of Sperry and Webb (1950) was used. A sample of blood was also simultaneously drawn from the volunteers and serum cholesterol was estimated by the method of Sperry and Webb (*loc. cit.*).

(b) *Effect of feeding different fats.*—The volunteers were then divided into three batches of two individuals each. One group was given 100 g. butter (equivalent to 85 g. of fat) daily for 10 days. The second group was given 85 g. of hydrogenated groundnut oil daily for 10 days, and the third group received 85 g. sesame oil daily for a similar period. Ten days after the high fat feed, sebum was collected as before and estimations carried out. At the time of this second collection of sebum, a sample of blood was also drawn and serum cholesterol estimated.

(c) *Study of sebum in pathological conditions.*—Four subjects were taken up for this study. Two of these were adult males suffering from nutritional oedema. The clinical features of these cases corresponded to the earlier description (Gopalan *et al.*, 1952). In these two subjects sebum was collected as usual and samples of blood were also obtained for serum cholesterol estimation. The two subjects were then placed on a high-protein diet for one month at the end of which period, their clinical condition had returned to nearly normal. Sebum was again

collected at the end of this treatment and at the same time blood samples obtained for serum cholesterol estimation.

The third subject of this study was a young male suffering from severe glossitis, angular stomatitis and ocular changes attributable to vitamin B-complex deficiency. The fourth subject was an adult female suffering from myxœdema, confirmed by the observed marked lowering of the basal metabolic rate. In these two latter subjects the sebum was collected only once on admission and at the same time samples of blood were obtained for serum cholesterol estimation.

The choice of the four subjects suffering from these three diseases was prompted by the following considerations. In nutritional œdema, marked lowering of serum cholesterol has been observed, while myxœdema is characterized by an elevation of serum cholesterol. In vitamin B-complex deficiency, skin changes characterized by dyssebacea are frequent, though the subject actually investigated here did not show any clinical evidence of dyssebacea. It was hoped that investigation of the skin in these conditions may throw some light on problems connected with cholesterol metabolism.

RESULTS.

The results of the investigation are presented in the Table. The cholesterol content of sebum of normal adults varied from 2.06 to 5.47 g. per cent; the ratio of free and ester fractions of cholesterol varied widely from individual to individual. The squalene content varied from 1.95 per cent to 4.16 per cent. There was no correlation between serum cholesterol, on the one hand, and sebum cholesterol or sebum squalene, on the other.

The feeding of butter and hydrogenated vegetable fat resulted in some increase in serum cholesterol while the feeding of sesame oil caused a fall in serum cholesterol. The changes in serum cholesterol observed with the feeding of different fats was not associated with any significant change in sebum cholesterol or squalene.

The low level of serum cholesterol in the two cases of nutritional œdema and the raised level of serum cholesterol in the case of myxœdema were not reflected by any changes in the sebum; the values for cholesterol and squalene in the sebum in these cases being not significantly different from the normal cases. Treatment with high-protein diet brought about a considerable increase in serum cholesterol levels in the two cases of nutritional œdema but the sebum cholesterol and squalene showed no significant parallel alterations. The values for sebum cholesterol and squalene in the case of vitamin B-complex deficiency were not abnormal.

DISCUSSION.

Mackenna *et al.* (1952) reported the mean squalene content of the skin of the forearm to be 5 per cent with a range from 3.3 per cent to 8.7 per cent. Boughton *et al.* (1957) obtained a mean value of 7.1 per cent for squalene for human arm sebum; these authors, however, pointed out that there were variations in the squalene content of sebum obtained from different parts of the same body. The wide variations in the squalene content of sebum obtained from different parts of

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THE EFFECT OF DIFFERENT FATS ON BLOOD COAGULATION.

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INTRODUCTION.

IN investigations of the pathogenesis of coronary heart disease, which are attracting world-wide interest today, factors influencing the development of atherosclerosis have received most attention. Recent evidence, however, suggests that the presence of pathological evidence of atherosclerosis of the coronary arteries need not invariably signify clinical coronary heart disease. For example, the incidence of degenerative changes in the coronaries, as revealed by post-mortem examination, in young American soldiers dying in the recent Korean War, was

found to be extremely high, and quite out of proportion to the incidence of coronary heart disease in the American population. It would appear, therefore, that in the development of coronary heart disease, factors other than those concerned in the pathogenesis of atherosclerosis may also be important. In this connection, factors influencing the coagulation of blood may deserve consideration.

The effects of different dietary fats on blood coagulation have been studied by a number of workers. The results of some of these studies have been contradictory. Duncan and Waldron (1949) demonstrated shortening of clotting time in dogs and in human beings after ingestion of cream. Fullerton *et al.* (1953) observed a significant reduction of clotting time and stypven time, in human volunteers, who were fed with 65 g. to 85 g. of animal fat. This was confirmed by O'Brien (1955), who also showed that feeding of different fats shortened the stypven time (O'Brien, 1956, 1957). Keys *et al.* (1957) observed that after all fatty meals, irrespective of the degree of saturation and the source of fat, whole blood clotting time was significantly shortened, but not after a control carbohydrate meal. Maclagan and Billimoria (1956) observed that ingestion of butter, but not margarine shortened the postprandial stypven or whole blood clotting time. Hall (1956) was unable to demonstrate any difference in clotting time or stypven time following alimentary lipemia, after a meal rich in butter. Merskey and Nossel (1957) observed significant reduction of stypven time, but obtained inconsistent results with regard to clotting time, following the ingestion of animal and vegetable fats.

In the present investigation, the effects of different lipids on whole blood clotting time and prothrombin time in a group of human volunteers have been studied.

MATERIALS AND METHODS.

1. *Effect of butter.*—Thirty healthy adult male volunteers between the age of 20 and 60 were the subjects of the investigation. Fifteen of these subjects were below 35 years and the remaining above 35 years of age. The investigations were carried out under identical conditions in all volunteers. The subjects were taken up for study on an empty stomach. After drawing a sample of fasting venous blood in a paraffinized syringe at 8.00 a.m., breakfast was given, which consisted of two slices of bread, 100 g. of butter (85 g. of fat allowing for 15 per cent moisture) and 8 oz. of tea. The volunteers were instructed to complete the breakfast within fifteen minutes. A second specimen of blood was drawn 3 hours later. The following tests were carried out on the two blood samples:

(a) *Coagulation time.*—Coagulation time was determined according to the method of Lee and White (1913) in ordinary (not silicone coated) Lee and White clotting tubes. One c.c. of blood was placed in each of the 4 test tubes (3×3/8 inch) in rapid succession. The tubes were kept in a water-bath at 37°C. and each tube was gently tilted once every half minute. The end-point was that which permitted complete inversion of the tube. The coagulation time recorded was the mean of readings obtained in the four tubes.

(b) *Stypven time.*—Russel viper venom was diluted 1 in 20,000. Equal volumes of platelet rich plasma, stypven and M/40 calcium chloride were incubated and coagulation time recorded. The tests were done in duplicate in all subjects,

(c) *Prothrombin time using rabbits' brain as thrombo-plastin.*—Instead of stypven, thromboplastin solution, prepared by the usual procedure from freshly killed rabbits' brain was used. The estimations were carried out in duplicate in 19 subjects.

(d) *Serum cholesterol.*—Total cholesterol was determined by the method of Abell *et al.* (1952) in 13 subjects.

(e) *Circulation time* was determined in 14 subjects using 4 c.c. of 20 per cent calcium gluconate, both during the fasting state and 3 hours after the ingestion of 100 g. of butter.

This investigation revealed that a number of volunteers responded to the high fat meal by showing significant changes in clotting time and stypven time, while the rest did not show any significant variation in clotting time.

2. *Reproducibility of results.*—To find out if repeated observation in any subject gives fairly constant results, the experiment was repeated in twelve out of the 30 volunteers, of whom 5 had shown significant changes in clotting time and 7 had not.

3. *Effect of isocaloric low fat diet.*—To investigate if the changes observed in 1 above were specifically due to fat, twelve out of the 30 volunteers were re-investigated with isocaloric low fat (fat content 6 g.) diet. The meal consisted of 6 oz. raw rice, 3 oz. pulses, 12 oz. vegetables, 8 oz. butter milk and 2 g. oil. Among the 12 volunteers, 9 had already responded to butter by increased coagulability. The results showed that there was no variation in clotting time or stypven time, following a low fat isocaloric meal.

4. *Effect of vegetable fats.*—The effect of vegetable fats was then investigated. Ten volunteers, who had previously shown increased coagulability after feeding of butter, were taken up for the investigation. Eighty-five g. of hydrogenated vegetable fat (dalda) mixed well within 5 oz. of wheat flour and made into 'chappattis' were given to the volunteers as breakfast and the changes in blood coagulability investigated.

The effect of coco-nut oil was investigated in ten volunteers, of whom seven had previously had butter and hydrogenated vegetable fat and the remaining three had had butter only. Coco-nut oil was administered in exactly the same manner as the hydrogenated fat in the form of 'chappattis'.

To verify that the variation in the responses to the three fats was not due to any changes occurring in the subjects with the passage of time, four volunteers who had responded differently to butter, hydrogenated fat and coco-nut oil were again investigated with butter; the sequence in which the fats were investigated in these four subjects being, butter, hydrogenated vegetable fat, coco-nut oil, butter. The response obtained with butter in the initial and final experiments was identical in all four volunteers.

RESULTS.

The results of the investigations are presented in Tables I, II and III.

1. *Effect of butter.*—When all the 30 subjects investigated with butter were considered together the average whole blood clotting time was 5' 18" before, and 4' 15" after the fat meal. This difference was found to be statistically significant,

even when allowance was made for the differences between the four readings on the same samples. When the subjects were considered individually, the differences between the whole blood clotting time before and after butter were statistically significant in 18 subjects and not significant in the remaining 12. The mean whole blood clotting time in these 18 cases was 5' 37" before and 3' 56" after the fat meal. Of these 18 cases, 10 were under 35 years and 8 above 35 years of age.

While the prothrombin time using rabbits' brain emulsion showed no significant change as a result of fat feeding in any of the subjects investigated, the stypven time showed significant reduction in the cases investigated. When all 30 cases were considered the mean stypven time was 14.6" before the fat meal and 13.0" after. This difference of 1.6" was found to be statistically significant ($t=3.80$, $P<0.001$). There was no correlation between the reduction in whole blood clotting time and in stypven time in the series.

There was no significant elevation of total serum cholesterol in the cases investigated, at the end of 3 hours after the fat meal. There was no significant change in the circulation time after fat meal in the 14 cases investigated.

2. *Reproducibility of results.*—The values for reduction of clotting time and stypven time obtained on repetition of the investigation, were found to be almost similar to the initial values in 6 of the 12 cases; in 4 of the remaining cases the difference between the two values was slight. The difference between the first and second set of values in the 12 cases investigated was found to be statistically not significant.

TABLE I.
Clotting time with different fats.

Subjects age, years.		REDUCTION IN CLOTTING TIME IN SECONDS:					Subjects age, years.		REDUCTION IN CLOTTING TIME IN SECONDS:				
		Butter.	Isocaloric low fat diet.	Hydro- genated fat.	Coco-nut oil.	Repetition of butter.			Butter.	Isocaloric low fat diet.	Hydro- genated fat.	Coco-nut oil.	Repetition of butter.
1	21	53*	..	0	..	..	16	35	116*	+10	+74	+68	90*
2	21	175*	..	..	..	..	17	37	29	..	..	..	..
3	22	+22	+3	..	..	..	18	37	17	..	..	..	..
4	22	86*	..	..	..	..	19	40	+6	..	..	..	..
5	23	47*	..	..	52	120*	20	43	+5	..	..	..	..
6	24	8	+5	..	..	..	21	45	99*	..	..	..	..
7	24	26*	+7	..	45	..	22	45	79*	..	+262	..	..
8	25	+17	..	..	..	..	23	47	63*	..	177*	+143	..
9	26	45*	+26	127*	75*	..	24	47	86*	..	7	83*	..
10	26	63*	5	5	+127	..	25	49	39*	+7	..	..	..
11	26	+11	3	..	..	..	26	50	+12	..	..	..	..
12	26	93*	0	82*	105*	120*	27	50	+18	..	..	..	..
13	27	49	..	..	..	..	28	52	173*	0	118*	..	..
14	34	157*	3	..	60	..	29	52	+24	..	..	..	..
15	34	84*	0	+90	+75	90*	30	61	116*	..	..	..	..

+ Sign shows that there was actually an increase in clotting time rather than reduction.
* Sign indicates that the values are statistically significant.

Effect of Different Fats on Blood Coagulation.

TABLE II.

Stypven time with different fats.

Subjects age, years.		REDUCTION IN STYPVEN TIME IN SECONDS:					Subjects age, years.		REDUCTION IN STYPVEN TIME IN SECONDS:				
		Butter.	Iso-caloric low fat diet.	Hydro- genated fat.	Coco-nut oil.	Repetition of butter.			Butter.	Iso-caloric low fat diet.	Hydro- genated fat.	Coco-nut oil.	Repetition of butter.
1	21	2.5	..	+1.0	..	..	16	35	9.0	1.0	0.0	+0.5	8.5
2	21	0.0	..	..	..	..	17	37	+0.5	..	..	..	..
3	22	0.0	0.0	..	..	..	18	37	+1.0	..	..	..	..
4	22	2.5	..	..	..	..	19	40	3.5	..	..	..	..
5	23	0.0	..	..	0.0	0.0	20	43	2.0	..	..	..	..
6	24	1.0	0.0	..	..	..	21	45	5.0	..	..	..	..
7	24	+0.5	0.0	..	+2.0	..	22	45	0.0	..	+2.0	..	..
8	25	+0.5	..	..	..	..	23	47	0.0	..	+2.5	0.0	..
9	26	2.5	0.0	0.0	+1.5	..	24	47	5.0	..	1.5	1.5	..
10	26	1.0	1.0	1.0	0.0	..	25	49	0.0	0.0	..	..	..
11	26	0.5	0.0	..	..	..	26	50	0.0	..	..	..	..
12	26	0.0	0.5	0.0	+0.5	0.0	27	50	3.0	..	..	..	..
13	27	1.0	..	..	..	..	28	52	2.5	0.0	+1.5	..	..
14	34	3.0	0.0	..	0.0	..	29	52	2.5	..	..	..	..
15	34	0.5	1.0	+1.0	0.0	0.0	30	61	0.0	..	..	..	..

+ Sign indicates an increase in stypven time instead of reduction.

TABLE III.

Effect of high fat (butter) diet on prothrombin time (rabbits' brain emulsion), circulation time and total serum cholesterol.

PROTHROMBIN TIME IN SECONDS (19)*:		CIRCULATION TIME IN SECONDS (14)*:		TOTAL SERUM CHOLESTEROL (13)*:	
Initial.	3 hours after feed.	Initial.	3 hours after feed.	Initial.	3 hours after feed.
14.8	14.3	15.2	15.6	128 mg. per cent.	132 mg. per cent.

*Figures within the brackets indicate the number of cases investigated.

3. *Effect of isocaloric low-fat diet.*—In all 12 cases investigated there was no significant change in the whole blood clotting time or stypven time after feeding this diet. Nine of these 12 subjects had earlier responded to the feeding of butter by reduction of whole blood clotting time and stypven time. This would show that the changes in coagulability observed after the butter meal were specifically due to the fat.

4. *Effect of vegetable fats.*—Only 4 of the 10 volunteers who had responded positively to butter responded positively to hydrogenated vegetable fat also. The remaining 6 did not show any significant reduction in clotting time; only 3 of the

same ten volunteers who had responded positively to butter responded to coco-nut oil and 2 of these were cases that had responded previously to hydrogenated vegetable fat also.

DISCUSSION.

The technique adopted in the present investigation for the determination of clotting time and stypven time did not involve the use of silicone coated tubes and platelet poor plasma. [As Hall (*loc. cit.*) has pointed out, the use of silicone-coated tubes, by unduly minimising the contribution of platelet breakdown towards clotting, may be less representative of *in vivo* conditions than the techniques employed here. However, the results obtained here following the feeding of butter, are in line with the observations of Fullerton *et al.* (*loc. cit.*) and O'Brien (*loc. cit.*) who used silicone coated tubes in their investigations. The present investigation has also shown that the effects of feeding butter on the clotting time and stypven time in the same individuals on different occasions are nearly similar.

The work of Duguid and Robertson (1957) has shown that with continued high fat feeding 'tolerance' to the fatty meal might develop.

Most of the subjects investigated in the present study, however, were subsisting on diets which were either low or moderate in fat. Even so, the fact that only 18 of the 30 subjects studied exhibited reduction in clotting time after a butter meal, points to individual variability in this regard, the explanation for which is not clear.

The investigation failed to reveal any significant change in circulation time after high fat diet. This aspect of the investigation was prompted by the observation of Swank (1954) in hamsters, that 3 hours after a meal rich in cream, there was increased viscosity of blood.

The failure of isocaloric low fat diet to produce changes in clotting time and stypven time observed with butter would suggest that fat plays a specific rôle in these changes. With regard to prothrombint ime, it was observed that the prothrombin time using rabbits' brain emulsion did not reveal any significant change. On the other hand, the stypven time showed significant alteration after the butter meal. While the individual responses varied, the mean value obtained after the butter meal was significantly lower than the mean initial value. This was not so with regard to the other fats. The reduction of stypven time after a butter meal has been attributed among other factors to increased presence in the blood of a phospholipid material, probably phosphatidyl ethanolamine (P.E.) after a butter meal. Highly active P.E. has been isolated from butter (MacLagan and Billimoria, *loc. cit.*). (The reason for the failure to observe any reduction in the prothrombin time using rabbits' brain emulsion may be attributed to the high content of the phospholipid material already present in the emulsion.

Differences with regard to changes in clotting time and stypven time were observed with different fats in the present investigation. Butter, hydrogenated vegetable fat and coco-nut oil could be placed in the descending order with regard to their effect on whole blood clotting time. Only butter produced a significant reduction in stypven time. Keys *et al.* (*loc. cit.*), however, observed no differences between different fats with regard to reduction in clotting time,

while O'Brien (*loc. cit.*) noticed differences in stypven time reduction between different fats, which he believed to be attributable to differences in the chain length of the fatty acids present in them.

Macfarlane *et al.* (1941) and Robinson and Poole (1956) had attributed the effect of dietary fats on blood clotting to lipæmia and increased chylomicra in blood. MacLagan and Billimoria (*loc. cit.*), however, had shown that the degree of lipæmia and the chylomicran count following on different fats were nearly similar. They found that in spite of the same degree of lipæmia following on a butter or a margarine feed, there was significant reduction of clotting time after butter but not after margarine.

A satisfactory explanation for the differences observed between different fats in the present investigation is not possible at this stage. It would appear from the results, that differences between the different fats with regard to the degree of saturation or the chain length of their component fatty acids may not account for the differences in their effects on clotting time or stypven time. It is a moot question as to how far the *in vitro* observations obtained under these experimental conditions, are applicable to human *in vivo* conditions. The practical significance of the observations in the present investigation will depend on the answer to this question.

SUMMARY.

1. The effects of different types of fat on blood coagulation of human volunteers has been studied.
2. Following an intake of 85 g. of butter fat, the mean clotting time and stypven time of 30 volunteers were significantly less than the fasting values. However, prothrombin time using rabbits' brain emulsion, circulation time and serum cholesterol were not influenced by the high intake of butter.
3. Hydrogenated vegetable fat and coco-nut oil did not influence stypven time.
4. Butter, hydrogenated vegetable fat and coco-nut oil could be placed in the descending order with regard to their effect on whole blood clotting time.
5. The significance of the results has been discussed.

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EFFECT OF SOME DIETARY FACTORS ON SERUM CHOLESTEROL.

BY

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INTRODUCTION.

EPIDEMIOLOGICAL observations pointing to correlation between serum cholesterol concentration and incidence of coronary atherosclerosis in population groups (Keys, 1954; Keys and Anderson, 1955) have stimulated wide interest in the investigation of factors influencing serum cholesterol concentration. Among the various factors now known to influence serum cholesterol level, diet has attracted special attention. Since cholesterol can be synthesized endogenously (Srere *et al.*, 1950; Gould *et al.*, 1951), the level of dietary cholesterol is now believed to play a relatively unimportant rôle in determining serum cholesterol concentration (Keys and Keys, 1954; Keys and Anderson, 1955; Keys *et al.*, 1956). On the other hand, considerable evidence has accumulated to show that the level of fat in the diet may be an important factor in this respect (Bronte-Stewart *et al.*, 1955; Keys *et al.*, 1956; Mayer *et al.*, 1954). The effects of different types of fat on serum-cholesterol concentration have been recently investigated and discussed by a large number of workers (Kinsell, 1952, 1954; Ahrens *et al.*, 1954, 1957; Bronte-Stewart *et al.*, 1956; Beveridge *et al.*, 1955, 1956; Keys *et al.*, 1957; Malmros, 1957).

The effects of dietary constituents, other than fat, on serum cholesterol, have attracted less attention. There are evidences indicating the effects of methionine, cystine and choline (Mann *et al.*, 1953), of some vitamins (Altschul *et al.*, 1955; Felch and Dotti, 1949; Chakravarti *et al.*, 1957) and of variation in caloric intake on serum cholesterol. However, the rôle of dietary factors other than fat on serum cholesterol concentration needs further investigation. An important objection to epidemiological observations pointing to a relationship between fat intake and serum

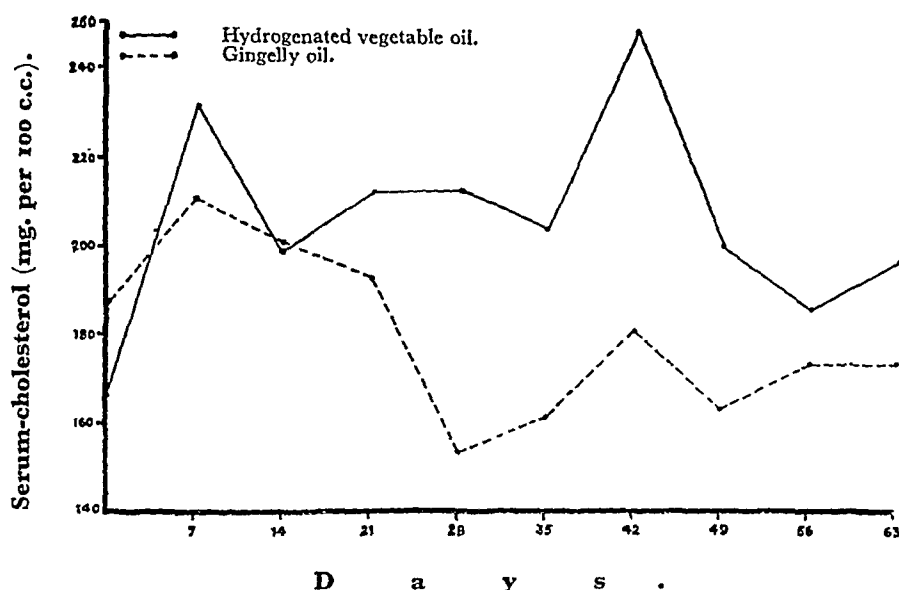
They were, thus on a low protein, adequate calorie diet. The animals were maintained on their respective diets for 35 days, at the end of which period serum-cholesterol determinations were repeated in all monkeys.

Monkeys in all the experiments were kept in individual cages throughout the experiment and were not released for exercise. This was to ensure uniformity with regard to the factor of physical exercise.

RESULTS.

The results of the above experiments are presented in Graphs 1 and 2 and Table III.

GRAPH 1.

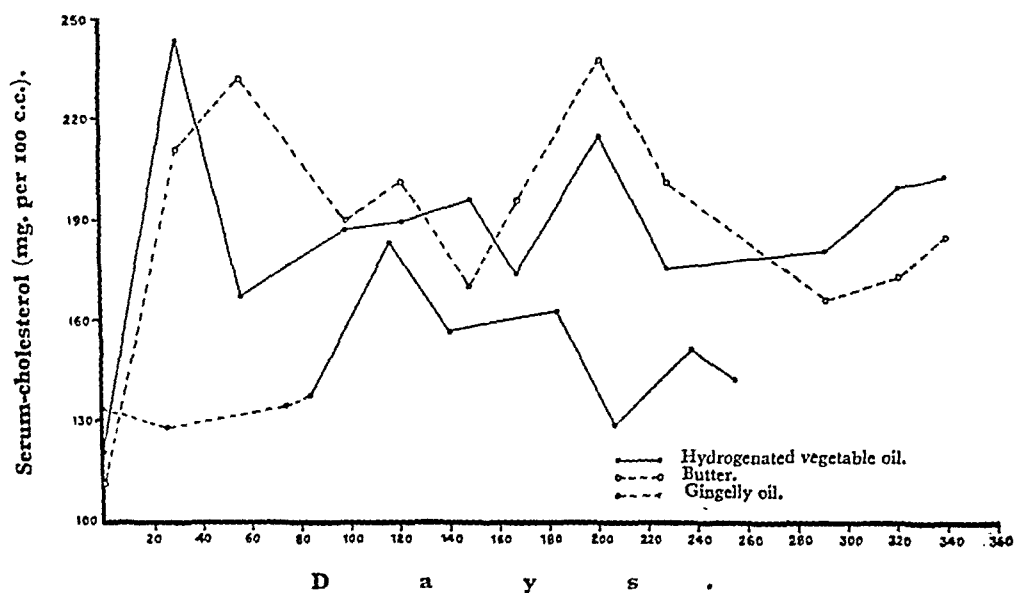


Serum-cholesterol levels in volunteers maintained on diets providing fats as hydrogenated vegetable oil or gingelly (sesame) oil.

Experiment 1.—The results indicate that the type of fat in the diet has an influence on serum-cholesterol level. Human volunteers receiving hydrogenated vegetable fat showed a marked rise of serum cholesterol at the end of one week. Though serum-cholesterol concentration was not maintained at this same high level throughout the period of feeding, the level was continuously maintained well above the starting 'basal' level throughout the experimental period. On the other hand, volunteers receiving sesame-oil showed a small rise in the serum-cholesterol values at the end of one week but by the fourth week, the values had returned to the levels below the starting value and continued to remain low throughout.

Experiment 2.—In the case of the monkeys the difference in the response of serum cholesterol between the groups fed butter and hydrogenated vegetable fat (I and II) on the one hand, and the group fed sesame-oil (III) on the other, was even more striking than in the case of human volunteers. This difference should

GRAPH 2.



Serum-cholesterol levels in monkeys maintained on diets providing fat as butter, hydrogenated vegetable oil, or gingelly (sesame) oil.

TABLE III.

Effect of protein and calorie deficiency on serum cholesterol in monkeys.

Group.		Initial serum cholesterol, mg./100 c.c.	Serum cholesterol at the end of 35 days, mg./100 c.c.	Actual reduction in serum cholesterol, mg./100 c.c.
Low-calorie low-protein	1	165	110	55
	2	160	95	65
	3	175	110	65
	4	175	115	60
	5	170	110	60
	6	170	85	85
High-calorie low-protein	1	190	100	90
	2	125	105	20
	3	165	140	25
	4	135	110	25
	5	160	105	55
	6	170	125	45

The difference in reduction on serum cholesterol level between low-calorie low-protein and high-calorie low-protein groups is not significant.

be considered to possess greater significance than that observed in the human volunteers for the reason that apart from the type of fat consumed, uniformity regarding the intake of calorie and nutrients and the factor of physical exercise in all the groups had been ensured. Even with regard to the pre-experimental diet,

it may be considered that there was greater uniformity in the case of the monkeys than in human volunteers, considering that they were all inhabitants of the same forest with equal and abundant opportunities to obtain the same kind of foods. It may be pointed out, however, that the level of fat fed to the monkeys was of a higher order than in human volunteers.

It was observed in the case of monkeys as in the case of human volunteers that the sharp rise in serum-cholesterol concentration occurring within the first few days after initiating feeding with large quantities of butter and hydrogenated vegetable fat was not sustained at the same high level throughout the experiment. The levels tended to come down but were always well above the starting values throughout the period of the experiment. It was interesting that this increase over the starting level was maintained for as long a period as 49 weeks over which the experiment lasted. On the other hand, with continued high fat feeding the rise in serum cholesterol was not progressive. For example, the values in the 39th week of the experiment were not significantly higher than in the 23rd or 14th week. This might indicate some adaptation on the part of the organism to high fat feeding.

The monkeys which had received high sesame oil diet showed no alterations in serum cholesterol levels over the 12 weeks of experimental period, but when they were switched over to high hydrogenated vegetable fat diet, there was a sharp increase in serum cholesterol concentration in the first few weeks. The serum cholesterol concentration tended to come down later and at the end of 18 weeks the level was almost the same as at the start. Throughout the rest of the experiment which lasted for 7 more weeks, the serum cholesterol concentration remained lower than in groups I and II (*vide* Graph 2). It appeared that pre-treatment with sesame oil for 12 weeks had to some extent inhibited the rise in serum cholesterol following on hydrogenated fat feeding.

Experiment 3.—Animals on the low-calorie low-protein diet as well as animals on the high-calorie low-protein diet showed significant reduction in serum cholesterol concentration. The reduction observed in animals of the low-protein low-calorie group was greater, but the difference between the two groups in this respect did not appear significant. This would indicate that both calorie and protein restriction might contribute to the reduction of serum cholesterol levels. It must be pointed out, however, that the protein restriction in this experiment was severe, the diet containing only 3.5 per cent protein. Animals of both groups lost body-weight during the experiment, but the loss in weight in animals on the low-protein low-calorie group was four times as great as in animals on the low-protein high-calorie group. The differences in the reduction in serum cholesterol between the two groups were much less than the differences in the reduction of body-weight between the two groups. It would appear, therefore, that the reduction in serum cholesterol brought about by severe protein deficiency is, in the main, not a reflection of reduction of body-weight.

DISCUSSION.

The observation that the effect of ingestion of fat on serum-cholesterol concentration depends on the type of fat, is in line with the findings of other workers

(Kinsell, 1952; Ahrens *et al.*, 1954; Beveridge *et al.*, 1955; Ahrens *et al.*, 1957; Keys *et al.*, 1957; Malmaros, 1957). Various reasons have been suggested to explain the differences between the fats with regard to their effects on serum cholesterol, such as differences in sitosterol content; in the essential fatty acid content; in the content of total unsaturated fatty acids; and possibly to the presence of unknown factors. The exact explanation should await further investigation. Of the various hypotheses suggested in this regard the rôle of essential fatty acids would seem to deserve particular attention. Previous investigations had shown that the content of essential fatty acids was roughly eight times higher in sesame-oil than in the hydrogenated vegetable fat used in the experiment. Though ground-nut oil, the original source of the hydrogenated vegetable fat used here, contains essential unsaturated fatty acids to the extent of nearly 27 per cent, presumably they are mostly destroyed in the process of hydrogenation. The difference between hydrogenated vegetable fat and sesame-oil cannot be attributed to differences in sitosterol content of a vegetable oil, and secondly, unlike in certain animals, sitosterol has not been shown to play a significant rôle in cholesterol absorption in the human subjects (Peterson, 1951).

The observations on monkeys in the present investigation were carried out for 49 weeks, which is much longer than the periods of observation employed in most other investigations of this kind. It was noteworthy that the rise in serum-cholesterol concentration following feeding of butter or hydrogenated vegetable fat was maintained throughout this prolonged period. At the same time, the absence of a progressive increase in serum-cholesterol concentration, and the fact that the sharp rise in serum-cholesterol observed in the initial phase of the fat feeding was not sustained at the same high level, would indicate the ability on the part of the organism to adapt itself to some extent to the high intake of fat.

The diets of population groups between which significant differences in serum cholesterol concentration have been reported (Walker and Arvidson, 1954; Keys and Anderson, 1955) differ not only in their fat content but also in their protein content and possibly calorific value. The observation here that calorie and protein deficiency can bring about a reduction in serum cholesterol concentration indicates the need for caution in equating differences in serum cholesterol concentration between population groups to differences in fat intake alone. The finding that protein deficiency alone in the absence of calorie deficiency can induce reduction in serum cholesterol concentration is somewhat contradictory to the findings of Keys and Anderson (1957). It must be emphasized, however, that the protein deficiency employed here was of a much more severe degree than what obtained in the experiments of Keys *et al.* (1957). It is likely that marginal changes in protein intake may not significantly affect serum cholesterol concentration, and that apart from low fat intake, calorie deficiency rather than protein deficiency may be the important determinant of low serum cholesterol concentration among malnourished populations. Serum-cholesterol concentrations have been found to be low in nutritional oedema and in kwashiorkor at the height of the disease and tend to return to normal levels following on nutritional rehabilitation (Ramanathan, 1955). Structural changes in the adrenal cortex in monkeys in severe protein depletion have been demonstrated (Gopalan, 1956). It has also been shown that the adrenal cortex is an important site of synthesis of cholesterol

in the body (Le Roy *et al.*, 1957). These observations could provide the explanation for the observed lowering of serum cholesterol in severe protein deficiency.

SUMMARY.

1. The effect of feeding large quantities of different types of fat on serum-cholesterol concentration was investigated in a group of human volunteers and monkeys.

2. It was found that while butter and hydrogenated vegetable fat caused a sustained increase of serum cholesterol concentration, sesame oil had no such effect.

3. Diets deficient in proteins and/or calories were also found to reduce serum cholesterol concentration.

4. The significance of the above observations has been discussed.

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Ind. Jour. Med. Res., **46**, 3, May, 1958.

INFLUENCE OF COOKING ON THE NUTRITIONAL VALUE OF FOODS.

Part III.

VITAMIN A CONTENT OF SOME COOKED FOODS.

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THE results of analysis of the work done on the vitamin A content of the cooked foods are presented herein.

MATERIALS AND METHODS.

The details of the methods of preparing the various items of food preparations that have been analysed for their vitamins content have already been reported (Pai, 1957).

SUMMARY AND CONCLUSIONS.

1. Results of analysis of forty-two items of articles of foods for their vitamin A content in their pre-cooked and cooked stages are presented.

2. The percentage loss of vitamin A in all the preparations studied in the series varied between 10.5 and 70.0 per cent. In preparations of cereals the range was from 10.5 to 37.6 per cent; in vegetables it was from 34.3 to 39.5 per cent and in 'dahls' the loss ranged between 57.0 and 70.0 per cent.

Thanks are due to the Indian Council of Medical Research for their financial aid, to the Principal, Medical College, Baroda, for permission to work, to the Professor of Physiology for his keen interest; to Messrs. Glaxo Laboratories (India) Ltd., the Director, Central Drugs Laboratory, Calcutta, Messrs. Volkart Bros., Bombay, and Messrs. Ciba Ltd., Bombay, for their kind supply of some of the vitamin preparations used in the study.

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REGIONAL VARIATIONS AND ADEQUACY OF INDIAN DIETS : STATISTICAL EVALUATION OF DIET SURVEYS.

Part II.

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It has been pointed out in the earlier investigation (Taskar, 1956) that climatic and soil conditions influence, to a great extent, the dietary habits of the poor people. The regions having similar conditions have similar nutrient intake patterns. As the protective foodstuffs, such as milk and milk products are found in negligible amounts in poor class family diets, and as cereals and pulses form the most economic source of food, these variations in the intake patterns were attributed to the types of cereals consumed. The data on consumption of types of cereals and pulses for each of the diet survey considered in the previous study are available. It is, therefore, intended in the present investigation to re-examine the classification of States by considering these data.

MATERIAL AND METHODS.

The information regarding the intake of rice, wheat, millets, other uncommon cereals and pulses from the diet surveys presented in the Special Report (1951) of the Indian Council of Medical Research, was used for the purpose of analysis. As there is no information on the types of cereals consumed in the diet surveys from Bihar, the total number considered in the present investigation comes to 753.

The method and basis for studying the comparisons between States have already been stated in the earlier investigation (Taskar, *loc. cit.*).

It was found that when the number of variables are five or less the method of finding out the linear components, discovered by Truman Kelly (1935) was

much easier and useful. The main principle behind the method is of rotating the axes, taken two at a time, by an angle θ given by:

$$\tan 2\theta = \frac{2 \operatorname{cov}(x_1, x_2)}{V(x_1) - V(x_2)}$$

where $\operatorname{cov}(x_1, x_2)$, $V(x_1)$, and $V(x_2)$ are the covariance and variances of the chosen pair of variables. Formulæ (1) to (7) provide the values of the new matrix. This process is to be continued until all the covariances are made negligibly small, at which point the final variables constitute the n -components. The formulæ are:

$$Y_1 = x_1 \cos \theta + x_2 \sin \theta \quad \dots (1)$$

$$Y_2 = -x_1 \sin \theta + x_2 \cos \theta \quad \dots (2)$$

$$V(Y_1) = V(x_1) \cos^2 \theta + V(x_2) \sin^2 \theta + 2 \operatorname{cov}(x_1, x_2) \sin \theta \cos \theta \quad \dots (3)$$

$$V(Y_2) = V(x_1) \sin^2 \theta + V(x_2) \cos^2 \theta - 2 \operatorname{cov}(x_1, x_2) \sin \theta \cos \theta \quad \dots (4)$$

$$\operatorname{cov}(Y_1, Y_2) = \operatorname{cov}(x_1, x_2) (\cos^2 \theta - \sin^2 \theta) - [V(x_1) - V(x_2)] \sin \theta \cos \theta \quad \dots (5)$$

$$\operatorname{cov}(Y_1, x_3) = \operatorname{cov}(x_1, x_3) \cos \theta + \operatorname{cov}(x_2, x_3) \sin \theta \quad \dots (6)$$

$$\operatorname{cov}(Y_1, x_4) = \operatorname{cov}(Y_1, x_5); \operatorname{cov}(Y_1, x_5) \text{ etc. of the same type.}$$

$$\operatorname{cov}(Y_2, x_3) = -\operatorname{cov}(x_1, x_3) \sin \theta + \operatorname{cov}(x_2, x_3) \cos \theta \quad \dots (7)$$

$$\operatorname{cov}(Y_2, x_4); \operatorname{cov}(Y_2, x_5); \operatorname{cov}(Y_2, x_5); \text{ etc. of the same type}$$

RESULTS AND DISCUSSION.

The consumption values of the various foodstuffs in different States are given in Table I. The standardized variables are given in Table II. The correlation

TABLE I.
Mean values of cereals, etc. by States.
(Average per capita intake.)

Names of States.	Rice, oz.	Wheat, oz.	Millets, oz.	Other cereals, oz.	Pulses, oz.
Ajmer	<i>Nil</i>	30.07	<i>Nil</i>	<i>Nil</i>	0.63
Assam	19.40	0.47	<i>Nil</i>	0.17	1.62
Bhopal	1.15	11.80	<i>Nil</i>	1.65	1.95
Bombay	3.91	4.14	0.79	3.36	2.65
Coorg	22.27	<i>Nil</i>	0.10	0.23	0.83
Delhi	<i>Nil</i>	13.30	8.70	1.77	5.07
Hyderabad ..	10.17	0.77	11.49	0.28	1.57
Kashmir	25.60	1.30	<i>Nil</i>	<i>Nil</i>	0.60
Madhya Pradesh ..	10.28	3.86	1.74	4.86	5.11
Madras	10.66	0.37	0.10	6.11	0.89
Mysore	2.05	<i>Nil</i>	24.55	<i>Nil</i>	1.80
Orissa	20.82	0.47	<i>Nil</i>	0.11	2.31
Punjab	3.95	9.68	<i>Nil</i>	9.91	3.87
Travancore	14.05	<i>Nil</i>	<i>Nil</i>	1.37	0.88
Uttar Pradesh ..	11.20	8.85	<i>Nil</i>	1.95	2.10
West Bengal ..	21.84	1.22	<i>Nil</i>	<i>Nil</i>	1.59
S.D.	7.543	4.102	4.896	5.483	2.000

matrix for which the set of uncorrelated variables are found is given in Table III. The sets of equations to transform the original variables are shown in Table IV. The values of the transformed variables which are uncorrelated and which are expressed in standard deviation units are given in Table V.

TABLE II.

Standardized mean values of cereals, etc., by States.

Names of States.	Rice, oz.	Wheat, oz.	Millets, oz.	Other cereals, oz.	Pulses, oz.
Ajmer ..	-1.4693	6.0155	-0.6060	-0.3620	-0.7299
Assam ..	1.1026	-1.2012	-0.6060	-0.3316	-0.2381
Bhopal ..	-1.3168	1.5620	-0.6060	-0.0610	-0.0714
Bombay ..	-0.9515	-0.3055	-0.4455	0.2506	0.2795
Coorg ..	1.4826	-1.3150	-0.5856	-0.3194	-0.6299
Delhi ..	-1.4693	1.9277	1.1709	-0.0398	1.4873
Hyderabad	-0.1208	-1.1268	1.7417	-0.3110	-0.2599
Kashmir ..	1.9245	-0.9980	-0.6060	-0.3620	-0.7466
Madhya Pradesh	-0.1071	-0.3739	-0.2506	0.5244	1.5090
Madras ..	-0.0563	-1.2237	-0.5851	0.7520	-0.6004
Mysore ..	-1.1975	-1.3150	4.4081	-0.3620	-0.1464
Orissa ..	1.2902	1.2010	-0.6060	1.4455	0.1105
Punjab ..	-0.9463	1.0451	-0.6060	-0.1126	0.8888
Travancore	0.3937	-1.3150	-0.6060	-0.0063	-0.6053
Uttar Pradesh	0.0155	0.8427	-0.6060	-0.3620	0.0036
West Bengal	1.4256	-1.0179	-0.6060	-0.3620	-0.2510

TABLE III.

Correlation matrix.

Foodstuff.	Rice.	Wheat.	Millets.	Other cereals.	Pulses.
Rice ..	..	..	..	..	..
Wheat ..	..	..	..	..	..
Millets ..	..	-0.3630	..	..	..
Other cereals	..	..	-0.1671	..	..
Pulses ..	..	..	-0.1339	-0.3632	-0.1975
	..	..	..	-0.1920	0.1709
	..	..	..	-0.1886	-0.0573
	..	..	..	..	0.1097
	..	..	..	..	..

TABLE IV.

Sets of equations for transforming original variables.

	x_1	x_2	x_3	x_4	x_5
Y_1 ..	0.389606	+1.073216	..	..	..
Y_2 ..	0.257798	+0.232565	..	..	..
Y_3 ..	0.733264	+0.565154	+1.037794	..	..
Y_4 ..	0.130993	-0.138359	+0.434718	+1.254140	..
Y_5 ..	..	..	+0.046708	-0.083342	+1.031139

TABLE V.

Mean values transformed variables.

Names of States.	Y_1	Y_2	Y_3	Y_4	Y_5
Ajmer	-1.4693	5.8835	0.3913	1.6049	-1.7754
Assam	1.1026	-0.8595	-0.6241	-0.5497	0.0644
Bhopal	-1.3168	1.1634	-0.6051	-0.4227	-0.4854
Bombay	-0.9515	-0.6986	-0.7786	-0.7498	0.1642
Coorg	1.4826	-0.8337	-0.5313	-0.3113	-0.2742
Delhi	-1.4693	1.4964	1.2847	0.4711	1.1324
Hyderabad ..	-0.1208	-1.2564	1.5143	-0.3583	-0.0206
Kashmir	1.9245	-0.3213	-0.3649	0.1298	-0.3776
Madhya Pradesh ..	-0.1071	-0.4430	-0.3747	0.2590	1.5384
Madras	-0.0563	-1.3352	-0.9063	-0.0442	-0.5472
Mysore	-1.1975	-1.8779	3.9603	-0.1590	0.1101
Orissa	1.2902	-0.7862	-0.5756	-0.4259	0.4494
Punjab	-0.9463	0.7529	-0.6208	1.4462	0.4991
Travancore ..	0.3937	-1.2579	-0.8332	-0.8591	-0.4095
Uttar Pradesh ..	0.0155	0.9105	-0.4289	0.2164	-0.1387
West Bengal ..	1.4256	-0.5370	-0.4981	-0.2474	0.0706

It will be seen from Table I that both rice and wheat are being consumed by the people from the various States, but the proportions of the two constituents in the diet vary from State to State. In Ajmer and Delhi there is no rice, while in Coorg, Mysore, Travancore-Cochin, wheat is not consumed. Similar is the case for millets and other uncommon cereals; when there is more of millets there is less of these cereals, and sometimes complete absence of one in preference to the other. The diets in all the States are supplemented with pulses to varying degrees. This is substantiated by the values of coefficients from the correlation matrix. The negative signs of the coefficients indicate that there is an inverse relation between the constituents, i.e., if one constituent in the diet increases the other is proportionately reduced. All the correlations are statistically significant, except millets—pulses (-0.057). It is interesting to note that the intake of pulses decreases as the consumption of rice increases, while it is directly proportional in the case of wheat. These peculiarities may arise from the fact that the surveys are carried out mainly on poor families where the total family earnings are spent in buying the main items of subsistence that are readily available.

The values D^2 given in Table VI indicate that thirteen States arrange themselves into three distinct groups, as in Table VII.

It is interesting to find from Tables I and VII that this grouping of States is similar to that given in the earlier communication, i.e., the first group composed of States where people consume more rice, while in the second wheat is found to be the most popular cereal, and in the third group of States, in addition to both rice and wheat, millets and other uncommon cereals are also consumed to an appreciable extent. This similarity in the grouping points out that, more or less, a correct classification of States with respect to intake patterns can be achieved by considering the chief type of cereals consumed by the people. Mysore and Hyderabad, in which millets are consumed in very high proportions, fall far away from other States. Similarly, Ajmer is farther away, as being a famine camp area, the people were given only wheat and no other cereals.

TABLE VI.

Values of D^2 (arranged in increasing order).

Ajmer.		Assam.		Bhopal.		Bombay.	
Bhopal	29.07	Orissa	0.21	Uttar Pradesh	2.40	Madras	2.23
Delhi	29.79	West Bengal	0.32	Bombay	4.16	Travancore	2.47
Uttar Pradesh	32.21	Coorg	0.33	Punjab	4.77	Madhya Pradesh	3.85
Punjab	32.61	Travancore	1.03	Delhi	7.12	Bhopal	4.16
Bombay	54.27	Kashmir	1.69	Madras	8.07	Assam	4.32
Kashmir	54.72	Madras	2.28	Travancore	8.66	Uttar Pradesh	4.67
Madhya Pradesh	55.26	Bombay	4.32	Assam	9.04	Orissa	5.26
West Bengal	57.23	Madhya Pradesh	4.53	West Bengal	10.26	West Bengal	5.26
Madras	60.02	Uttar Pradesh	4.98	Orissa	10.76	Coorg	6.02
Coorg	60.61	Hyderabad	6.27	Coorg	11.47	Hyderabad	6.39
Hyderabad	60.99	Bhopal	10.26	Hyderabad	11.89	Punjab	6.45
Assam	61.14	Punjab	10.97	Kashmir	12.00	Kashmir	7.06
Orissa	62.11	Delhi	17.99	Ajmer	13.08	Delhi	9.65
Travancore	63.91	Mysore	27.50	Mysore	29.07	Mysore	11.77
Mysore	79.72	Ajmer	61.14		30.53	Ajmer	24.26
							54.27
Coorg.		Delhi.		Hyderabad.		Kashmir.	
West Bengal	0.22	Punjab	5.84	Travancore	6.18	West Bengal	0.66
Assam	0.33	Bhopal	7.12	Madras	6.25	Coorg	0.69
Orissa	0.58	Uttar Pradesh	7.16	Assam	6.27	Orissa	1.66
Kashmir	0.69	Madhya Pradesh	8.58	Bombay	6.45	Assam	1.69
Travancore	1.78	Hyderabad	11.47	Orissa	6.81	Travancore	4.42
Uttar Pradesh	2.91	Bombay	11.47	West Bengal	6.98	Uttar Pradesh	5.23
Madras	5.50	West Bengal	17.34	Coorg	7.00	Madras	5.30
Madhya Pradesh	6.32	Orissa	17.56	Madhya Pradesh	7.04	Madhya Pradesh	7.83
Bombay	6.39	Madras	17.90	Mysore	7.59	Hyderabad	8.95
Hyderabad	7.00	Assam	17.99	Uttar Pradesh	8.83	Bombay	9.65
Bhopal	11.89	Travancore	19.69	Kashmir	8.95	Punjab	11.97
Punjab	12.11	Kashmir	19.94	Delhi	11.47	Bhopal	13.08
Delhi	20.03	Coorg	20.03	Bhopal	12.00	Delhi	19.94
Mysore	28.62	Mysore	20.06	Punjab	12.84	Mysore	31.20
Ajmer	60.61	Ajmer	29.79	Ajmer	60.99	Ajmer	54.72
Madhya Pradesh.		Madras.		Mysore.		Orissa.	
Orissa	3.77	Travancore	0.90	West Bengal	7.12	Assam	0.21
Bombay	3.85	Bombay	2.23	Hyderabad	7.59	West Bengal	0.26
Assam	4.53	Assam	2.28	Delhi	20.06	Coorg	0.58
Uttar Pradesh	4.66	Coorg	2.91	Madhya Pradesh	24.25	Kashmir	1.66
Punjab	4.69	Orissa	3.36	Bombay	24.26	Travancore	2.02
West Bengal	4.78	West Bengal	3.42	Madras	25.73	Madras	3.36
Madras	5.52	Kashmir	5.30	Travancore	26.65	Madhya Pradesh	3.77
Travancore	6.17	Uttar Pradesh	5.51	Assam	27.50	Bombay	5.26
Coorg	6.32	Madhya Pradesh	5.52	Orissa	28.14	Uttar Pradesh	5.28
Hyderabad	7.04	Hyderabad	6.25	Coorg	28.62	Hyderabad	6.81
Kashmir	7.83	Bhopal	8.07	Uttar Pradesh	28.71	Punjab	10.88
Bhopal	8.58	Punjab	8.54	Bhopal	30.53	Bhopal	11.47
Delhi	8.66	Delhi	17.90	Punjab	30.78	Delhi	17.56
Mysore	24.25	Mysore	25.73	Kashmir	31.20	Mysore	28.14
Ajmer	55.26	Ajmer	60.02	Ajmer	79.72	Ajmer	62.11

TABLE VI—(Concl'd.).

Punjab.		Travancore.		Uttar Pradesh		West Bengal.	
Uttar Pradesh	2.91	Madras ..	0.90	Bhopal ..	2.40	Coorg ..	0.22
Madhya Pradesh	4.69	Assam ..	1.03	Punjab ..	2.91	Orissa ..	0.26
Bhopal ..	4.77	Coorg ..	1.78	West Bengal ..	4.35	Assam ..	0.32
Delhi ..	5.84	Orissa ..	2.02	Madhya Pradesh	4.66	Kashmir ..	0.66
Bombay ..	7.06	West Bengal ..	2.30	Bombay ..	4.67	Travancore ..	2.30
Madras ..	8.54	Bombay ..	2.47	Assam ..	4.98	Madras ..	3.42
West Bengal ..	10.36	Kashmir ..	4.42	Kashmir ..	5.23	Uttar Pradesh	4.35
Orissa ..	10.88	Madhya Pradesh	6.17	Orissa ..	5.28	Madhya Pradesh	4.78
Assam ..	10.97	Hyderabad ..	6.18	Coorg ..	5.50	Bombay ..	6.02
Kashmir ..	11.97	Uttar Pradesh	6.24	Madras ..	5.51	Hyderabad ..	6.98
Travancore ..	12.02	Bhopal ..	9.04	Travancore ..	6.24	Mysore ..	7.12
Coorg ..	12.11	Punjab ..	12.02	Delhi ..	7.16	Punjab ..	10.36
Hyderabad ..	12.84	Delhi ..	19.69	Hyderabad ..	8.83	Bhopal ..	10.76
Mysore ..	30.78	Mysore ..	26.65	Mysore ..	28.71	Delhi ..	17.34
Ajmer ..	32.61	Ajmer ..	63.91	Ajmer ..	32.31	Ajmer ..	57.23

TABLE VII.

Group I.	Group II.	Group III.
Assam Coorg Kashmir Orissa Travancore -Cochin West Bengal	Bhopal Delhi Punjab Uttar Pradesh	Bombay Madhya Pradesh Madras

The first two roots of the covariance matrix of the transformed variates are $\lambda_1 = 62.86$ and $\lambda_2 = 30.13$ adding up to 92.99. These two roots account for 84 per cent of the total variance. The other three roots contribute very little to the discrimination of the various States. Thus, a two dimensional pictorial representation of the States with respect to the first two canonical variates can, therefore, give a good idea of the configuration in the five dimensional space.

The canonical variates and mean values of the variates for all the States are given in Tables VIII and IX.

TABLE VIII.

Rice.	Wheat.	Millets.	Other cereals.	Pulses.
1. -0.352	0.883	0.044	0.269	-0.150
2. -0.471	-0.217	0.845	0.028	0.127

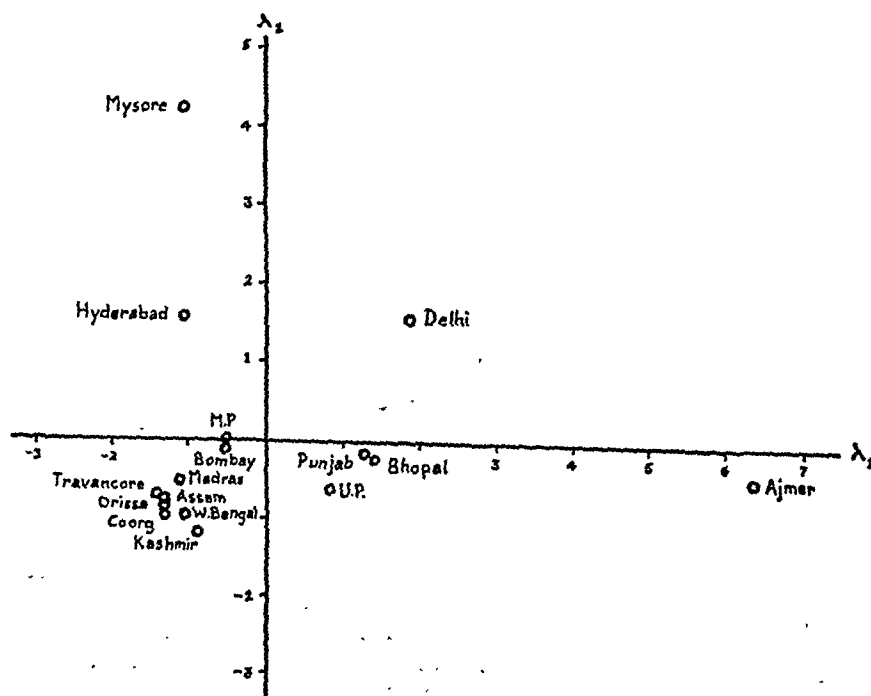
The components given in Table VIII are arranged in order of their capacity, as determined by the variation absorbed, in discriminating between the States. A close examination of the coefficients of each of the two components, reveals that wheat has the highest and rice has the lowest scores on the first component, while on the second component the millets have the highest and rice the lowest scores.

It is possible, therefore, to classify the States on the basis of consumption of rice, wheat and millets. By knowing the quantities of rice and wheat consumed the States can be divided into two groups which can be further sub-divided on the basis of consumption of millets. The values λ_1 (x-axis) and λ_2 (y-axis) are plotted to get a pictorial representation of the States as shown in the Graph.

TABLE IX.

		λ_1	λ_2
Ajmer	..	6.43	-0.43
Assam	..	-1.33	0.87
Bhopal	..	1.42	-0.22
Bombay	..	-0.52	-0.06
Coorg	..	-1.32	-1.01
Delhi	..	1.85	1.61
Hyderabad	..	-1.09	1.60
Kashmir	..	-0.89	-1.19
Madhya Pradesh	..	-0.33	0.03
Madras	..	-1.13	-0.52
Mysore	..	-1.12	4.33
Orissa	..	-1.36	-0.88
Punjab	..	1.29	-0.15
Travancore-Cochin	..	-1.45	-0.69
U.P.	..	0.86	-0.58
West Bengal	..	-1.08	-0.97

GRAPH.



SUMMARY.

The data on 753 diet surveys from different parts of India were analysed. It was found that it is possible to explain the differences in the intake patterns of nutrients on the basis of consumption of types of cereals and millets.

The authors have great pleasure in acknowledging their indebtedness to Dr. V. N. Patwardhan, Director, Nutrition Research Laboratories, for his valuable help and advice in the preparation of this paper.

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STUDIES ON THE EFFECT OF GERMINATION ON STREPO- GENIN CONTENTS OF PULSES.

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It was reported by Albanese and Irby (1943) and Bower and Berg (1943) that chickens, mice and rats grew more rapidly when they were fed intact protein than when they were fed a mixture of synthetic amino acids which constituted the same protein. This suggested the presence of a growth-promoting factor in intact protein which was different from the amino-acid composition of the protein molecule. Sprince and Woolley (1945) showed that the growth-promoting factor

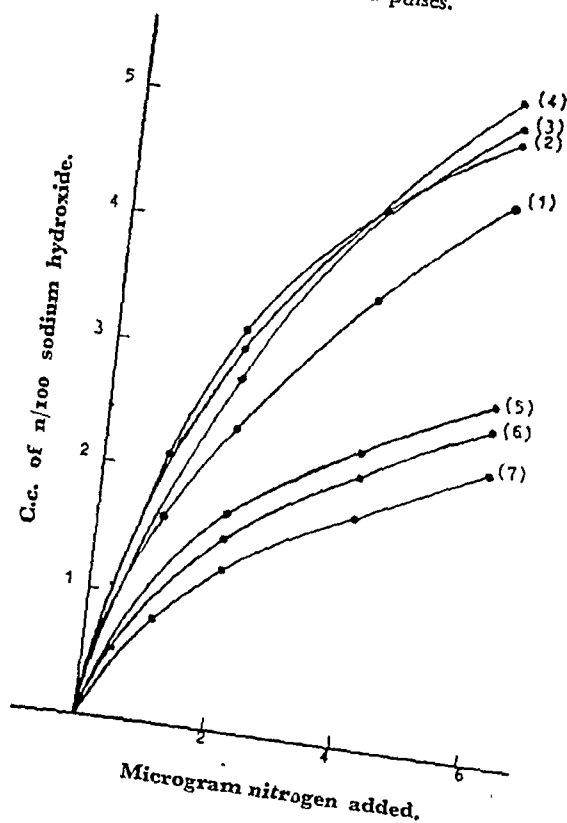
was peptide in nature, formed the integral part of the protein molecule and was liberated by tryptic digestion. They have described the factor as 'strepogenin' and estimated the strepogenin activity of various proteins by observing the growth-promoting activity of tryptic digests of protein on *Lactobacillus casei*. Wright and Skeggs (1944) observed strepogenin activity in tryptic digests of casein. Krishna-murthy and De (1951) reported that some pulses contain more strepogenin than casein. As pulses form an important ingredient of our daily diet, it was considered of interest to study the strepogenin activity of different varieties of pulses before and after they were germinated.

EXPERIMENTAL.

Germination of the seed.—Five g. of clean and healthy leguminous seeds (pulses) were taken in sterilized Petri-dishes, 3 c.c. of sterile distilled water was added and the dish kept away from direct sun light. Two c.c. of sterile water was added on the second day of germination and on the third day of germination the seeds were ground in a glass mortar, and transferred in a 250-c.c. Erlenmeyer flask with 50 c.c. re-distilled water.

Five g. of the powdered dry pulse and 5 g. of casein were transferred in separate 250-c.c. flasks. Fifty c.c. re-distilled water were added to each flask

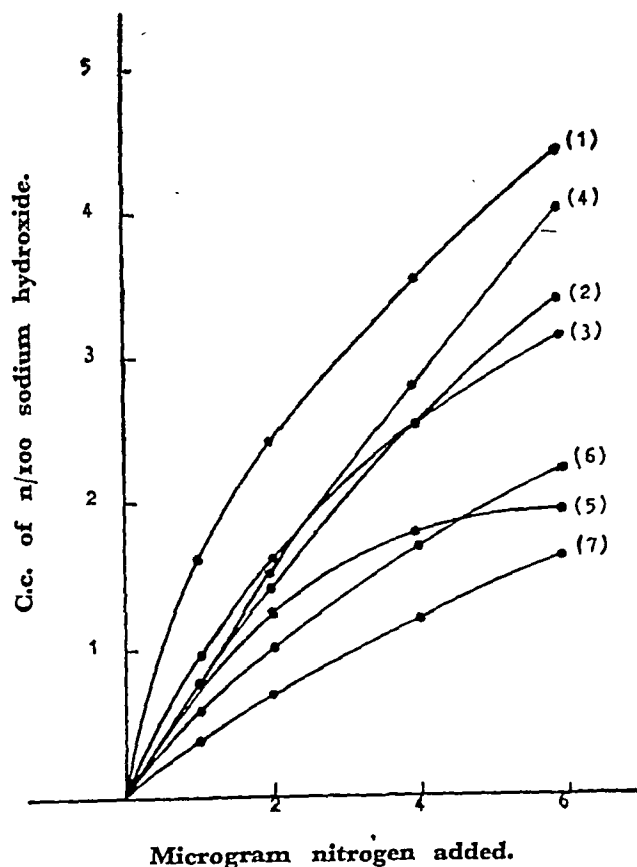
GRAPH 1.
Ungerminated pulses.



and strepogenin concentrates were prepared according to the method of Louise *et al.* (1948) with some modifications. pH of the contents of each flask was brought to 8.4 with 10 N sodium hydroxide, 100 mg. of trypsin followed by 75 c.c. of borate buffer of pH 8.4 added, the contents mixed, 1 c.c. toluene added, flasks plugged, incubated for 20 hours at 37°C., pH brought to 7 with concentrated hydrochloric acid, filtered, precipitate discarded, 2.5 g. lead acetate added to the resulting filtrate. precipitate removed by filtration, pH adjusted to 9.5, precipitate again removed by filtration, the filtrate was saturated with sulphuretted hydrogen, lead sulphide added and precipitate removed by filtration, the filtrate concentrated under suction until all H_2S was removed, toluene added, plugged and kept in refrigerator.

Nitrogen was estimated in the final concentrate by micro-Kjeldahl method and the concentrate was suitably diluted with water so that 1 c.c. of the diluted solution was equivalent to 4 γ nitrogen. Concentrates equivalent to 1 γ , 2 γ , 4 γ , and 6 γ nitrogen were added to rimless test tubes (6" $\times$ 3/4") containing basal media as described by Landy and Dickens (1942) with the addition of 10 γ /c.c. of manganese chloride and strepogenin activity was assayed microbiologically using *Lactobacillus casei* as the test organism. Acid produced after incubation for 24 hours was titrated with N/100 sodium hydroxide using bromothymol blue as indicator. Results which are averages of four observations in each case are shown in Graphs 1 and 2.

GRAPH 2.
Germinated pulses.



DISCUSSION.

It will be seen from Graph 1 that *Phaseolus mungo*, *Phaseolus radiatus* and *Pisum sativum* have more streptogenin activity than casein, while *Lens esculenta*, *Cajanus indicus* and *Cicer arietinum* show lesser activity. It is interesting to note that *Phaseolus mungo*, *Phaseolus radiatus* and *Pisum sativum* also contain more essential amino acids per gramme of protein than the other pulses (Chatterjee *et al.*, 1956). After germination, streptogenin activity of all the pulses studied diminished considerably and the activity was less than casein. During the process of germination streptogenin possibly breaks up into substances which have less growth-promoting value.

SUMMARY.

1. Streptogenin concentrates were prepared from six varieties of common Indian pulses and streptogenin activity was determined by microbiological assay using *Lactobacillus casei* as the test organism and casein as standard of reference. Streptogenin activity was also assayed after the pulses were germinated.

2. It was observed that *Phaseolus radiatus*, *Phaseolus mungo* and *Pisum sativum* had more streptogenin activity than casein, while *Cajanus indicus*, *Lens esculenta* and *Cicer arietinum* showed less streptogenin activity as compared to casein.

3. After the germination for 48 hours streptogenin activity diminished considerably in all the pulses and the activity was less in all cases than the activity of casein.

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THE EFFECT OF RICE AND WHEAT DIETS ON GROWTH, REPRODUCTION AND LACTATION IN ALBINO RATS.

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It has been generally recognized that the areas having a high density of population happen to be the areas where rice is the staple article of diet. This has led many to believe that the rice diet probably is conducive to better fertility. According to Ranganathan (1954) an examination of census figures for population increase or of birth rates for various provinces of India or of other regions of world consuming rice or wheat as the staple cereal do not show any association between the latter and fertility. Mathen (1956) in a recent review has also expressed the same opinion.

It is possible that the data on human population may indicate the trend but cannot afford incontrovertible evidence that a particular diet *per se* has any effect on the fertility rates. Several factors other than diet can complicate the issue. It was, therefore, felt that animal experiments carried out under rigidly controlled conditions and uncomplicated by other factors may be of help in assessing the effect of the staple cereal in diet on the fertility rate. It is with this end in view that experiments reported here were conducted.

EXPERIMENTAL.

Twentyone day old, healthy albino rats, from five litters, were equally distributed into two groups according to litters in such a manner that both the groups included one male and one female from each litter. One group of animals was fed a diet in which rice was the cereal, while the other group was fed a diet containing an equal amount of wheat, the other dietary constituents being identical. The composition of the diets is given in Table I.

TABLE I.

Composition of the rice and wheat diets.

Item.				Rice diet.	Wheat diet.
1	Rice ..	..	..	57.0	..
1(a)	Wheat ..	..	..	..	57.0
2	Skimmed milk ..	..	..	4.5	4.5
3	Calcium phosphate ..	..	..	1.0	1.0
4	Oil (ground-nut) ..	..	..	1.5	1.5
5	Sprouted Bengal gram ..	..	..	9.0	9.0
6	Cabbage (fresh) ..	..	..	26.3	26.3

0.7 g. oil containing 400 I.U. vitamin A is smeared over the chapatties.

Analysis of dried diets:

Ash, per cent ..	..	3.1	1.8
Fat, per cent ..	..	5.7	6.0
Protein, per cent ..	..	10.6	15.1
Carbohydrate by diff., per cent ..	..	80.6	75.8
Calcium, per cent ..	..	0.53	0.51
Phosphorus, per cent ..	..	0.43	0.36
Iron, mg. per cent ..	..	9.11	12.28
Calories per 100 g. diet ..	..	416	417

Items 1 to 4 mentioned in Table I were mixed thoroughly and made into a dough from which flattened, unleavened, bread (chapatti) was made. After baking, the chapatties were smeared with 0.7g. oil in which vitamin A concentrate had been dissolved.

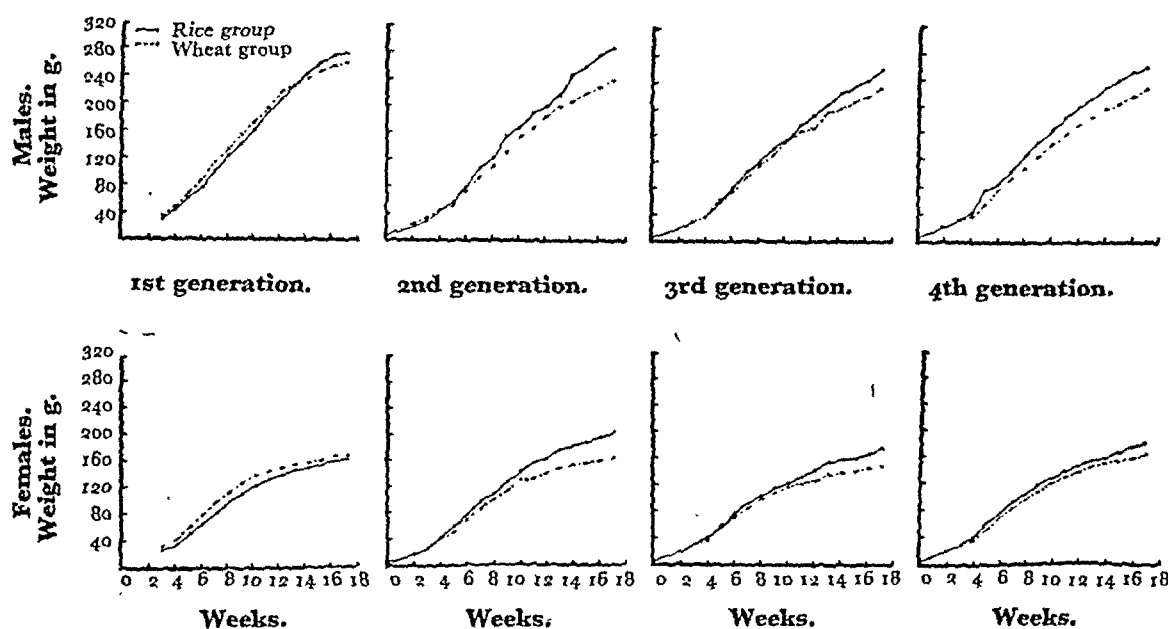
It will be seen from the composition of the diets that cereals formed about 57 per cent of the diets, which is normally found in the average dietary of people residing in so-called 'rice or wheat belts'. The incorporation of other ingredients ensured adequate intake of essential nutrients. The animals were weighed every week up to 120 days, when the males and females from the same litter were kept for mating, and continued on the respective diets. When the females showed advanced signs of pregnancy they were isolated but both the male and female were continued on the same diet. The pertinent records regarding prenatal period, litter size, birth weight of the young, were kept as described by Dikshit and Taskar, (to be published). Same males and females were mated and the breeding performance during different matings recorded. In order to avoid excessive stress, the females were permitted to suckle only six young; the 14th day weights of six young as suggested by Dikshit and Taskar (1956) and the percentage of young suckled till the weaning time, viz. 21 days were considered as criteria in the determination of lactation performance. The weight records of the animals born in the rice and wheat groups were kept till the 120th day and they were mated as stated above. Records of growth, breeding and lactation performance on the two diets were kept for three successive generations.

To determine whether the animals on the two cereal diets were comparable in every respect, estimations were carried out on the plasma protein, femoral length and bone ash content of animals on the two diets. These results are also included in this communication.

RESULTS AND DISCUSSION.

Growth rate of rats on rice and wheat diets.—In the Graph are given the growth curves of male and female rats born during different generations on the two cereal diets. The average weights of 120-day old rats for all four generations of rice group were 270.7 ± 6.42 g. for males and 178.9 ± 5.30 g. for females, whereas the corresponding figures for animals of the wheat group were 239.7 ± 5.22 g. and 157.8 ± 2.44 g., respectively. The differences between weights on rice and wheat diets were found highly significant.

GRAPH.



Growth rate of rats on rice and wheat diets during different generations.

It is clear, therefore, that the animals on the rice diet showed greater growth, in spite of the lower protein content of the rice diet. This may probably be due to the fact that rice protein is known to have a higher biological value than the protein of wheat.

The rate of growth of rats is determined by plotting the logarithm of weight against the reciprocal of time and expressed by an equation as suggested by Zucker *et al.* (1941). The constants 'K' of the growth curve of both male and female rats of the colony maintained by these Laboratories (Dikshit and Tasker, to be published) agree very closely with the values reported by Zucker, as shown in Table II. It is, therefore, surprising that the constants for the growth curves of rats in the rice and wheat groups are different from both the above. The slightly higher 'K' value is apparent both in the male and female rats on the two diets (Table II). Increases in the 'K' values for females *pari passu* with that of males leave the ratio of the constants for male and female unaffected. The reasons for the higher 'K' values are not clear.

TABLE II.

Constant K determining the growth rate of animals on rice and wheat diets.

Figures for:	MALES:			FEMALES:		K	MALES:
	Age in weeks range.	K	A	Age in weeks range.	K ₁	A ₁	K ₁ females.
Stock colony of Zuckers ..	4-17	3.65	470	4-50	2.84	270	1.29
Stock colony of Nutrition Research Laboratories ..	4-17	3.69	395	4-17	2.76	227	1.34
Rice group rats ..	4-17	4.28	451	4-17	3.44	283	1.25
Wheat group rats ..	4-17	4.19	398	4-17	3.28	254	1.28

Formulae: $\log W = -\frac{K}{t} + \log A$, where W=weight, t=time in weeks, A=weight approached asymptotically in the adult animal, and K=the slope of the line which characterizes the rate of growth.

REPRODUCTION PERFORMANCE ON RICE AND WHEAT RATION.

For assessing the reproduction performance of rats, the following four criteria are commonly employed (Schultze, 1955; 1955a; Dryden, Gleis and Hartman, 1956; and Dikshit and Taskar, to be published): (i) The percentage of fertile matings, (ii) the prenatal period or the interval between the placing of male and female together and the delivery of the young, (iii) the litter size, and (iv) birth weights.

TABLE III.

Reproduction performance of rats of rice and wheat diets of all four generations.

	RICE GROUP MATING NUMBER:				WHEAT GROUP MATING NUMBER:			
	1st	2nd	3rd	4th	1st	2nd	3rd	4th
1. Number of pairs kept for mating ..	29	26	25	25	28	28	27	27
Number of fertile matings ..	26	25	25	18	28	27	27	26
Per cent fertile matings ..	89.8	96.1	100.0	72.0	100.0	96.5	100.0	96.4
2. Prenatal period, days ..	24.6	26.0	27.4	25.3	25.4	25.3	25.3	24.8
3. Average weight of female when kept for mating, g.	180.7	198.1	214.6	233.2	158.5	186.2	192.9	203.6
4. Average litter size ..	6.85	7.56	6.48	5.72	8.07*	7.81	7.48*	7.19*
S.E. ..	±0.371	±0.383	±0.368	±0.452	±0.445	±0.256	±0.192	±0.419
5. Average birth weight, g.	4.79	4.86	4.84	5.10	4.96	4.95	5.01	5.00
S.E. ..	±0.092	±0.113	±0.083	±0.145	±0.104	±0.089	±0.025	±0.085

*Denotes significantly different from corresponding values in the rice groups.

A consolidated statement of the reproduction performance of rats kept for all four generations on the two cereal diets, has been set out in Table III. It will be seen from Table III that on the wheat ration, reproduction is superior to that in rats kept on rice diets. The differences were apparent even in the first generation. On the rice diet there were less fertile matings than on the wheat diet in the

first and fourth matings. There was, however, no appreciable difference in the prenatal period in the two groups. The better breeding performance on the wheat ration is clearly seen by the litter size. At any particular period of mating larger number of young were delivered in the wheat group. The differences were significant in the first, third and fourth matings.

The birth weight of the young rat depends upon: (i) the weight of the mother, (ii) the position of litter series, and (iii) the number of young delivered (Donaldson, 1924; Dikshit and Taskar, to be published). In view of this, it is somewhat surprising that despite the greater body-weight of females and smaller litter size in the rice group, the birth-weight of the young born in this group was smaller.

There was no significant difference in the male to female ratio of the young born in the two diet groups. In general, for every 100 females born there were 103 and 100.7 males born in rice and wheat groups, respectively.

Lactation performance.—It will be seen from Table IV that on the rice diet the lactation performance of rats was slightly inferior to that on the wheat diet. This is evident from the smaller percentage of young weaned by mother rats on the rice diet, in spite of the smaller number of suckling rats. The average weight of six young on the 14th day was also lower in the rice group, this being significant in the rats born in the first mating.

TABLE IV.

Lactation performance of female rats on rice and wheat diets.

	RICE GROUP MATING NUMBER:				WHEAT GROUP MATING NUMBER:			
	1st	2nd	3rd	4th	1st	2nd	3rd	4th
1. Total number of female rats	26	25	24	18	27	27	27	24
Number of young allowed to suckle	145	143	135	95	158	160	160	138
Average number of young allowed to suckle per female	5.58	5.72	5.63	5.28	5.85	5.93	5.93	5.75
2. Total number young weaned	126	137	120	90	152	151	156	137
Per cent young weaned	86.9	95.8	88.9	94.7	96.2	94.4	97.5	99.3
3. Average total weight of 6 young on the 14th day	110.8	111.6	121.4	120.5	120.8	119.1	121.6	123.4
Total number of groups	12	16	12	6	19	16	21	16

Serum protein content of young rats on rice and wheat diets.—Although as shown above some differences were observed in the growth, reproduction and lactation performance of rats on rice and wheat diets, in some other respects rats on these diets were comparable.

The serum protein content of 28- and 120-day old rats on rice diet was 5.43 and 6.71 g. per 100 c.c. serum, while the corresponding figures for the animals of the wheat group were 4.91 g. and 6.49 g. per cent, respectively. The differences, however, were not found statistically significant.

Femoral length and bone ash content of adult rats on rice and wheat diets.—According to Donaldson (*loc. cit.*) there is a direct relationship between the body-weight of rat and its body length which, in turn, is directly proportional to the femoral length. This observation has been confirmed in these laboratories. On the basis of this, one would expect the heavier rats in the rice group to have longer femoral bones. As shown in Table V the femoral lengths of four months old rats of the two groups were almost equal; the wheat-fed rats, if at all, have a slight edge in their favour. It is not unlikely, therefore, that the greater weight of rice group is due to a greater fat deposition in them.

There is no significant difference in the percentage of bone ash of the two groups as shown in Table V:—

TABLE V.

Femoral length and bone ash of 120 days old rats on the rice and wheat diets.

	R I C E :				W H E A T :			
	Male.		Female.		Male.		Female.	
	No of animals.		No of animals.		No of animals.		No of animals.	
Weight (average), g.	17	264.9	17	173.8	16	237.1	17	160.0
Femoral length, mm.	15	34.9	15	31.0	14	34.9	15	31.3
Bone ash, per cent	15	57.6	15	58.5	15	56.8	15	57.7

SUMMARY.

1. The growth of rats on rice diet was greater than on a comparable diet containing wheat as the cereal. The constant 'K' of the slope of growth curve on the two diets for both males and females was higher than that reported earlier. The probable significance is discussed in the text.
2. Reproduction and lactation performance of rats on rice diet was lower than that on wheat diet.
3. Bone ash and serum protein content of animals on rice diet were slightly though not significantly higher.

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Ind. Jour. Med. Res., **46**, 3, May, 1958.

NUTRITIVE VALUE OF DUCK EGG WHITE.

Part V.

SOME PROPERTIES AND COMPOSITION OF OVOMUCOIDS OF DUCK EGG AND HEN EGG WHITE.

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INTRODUCTION.

NARASINGA RAO AND PATWARDHAN (1954) have described certain marked differences in the biological activity of ovomucoids of the duck (DEW) and the hen egg white (HEW). The latter, when fed with the diet, did not inhibit the growth of rats, whereas the DEW ovomucoid markedly depressed it under comparable conditions. Vijayaraghavan and Narasinga Rao (1954) had earlier observed that HEW ovomucoid inhibited tryptic hydrolysis to a negligible extent when

the period of hydrolysis was extended to 24 and 120 hours as against the sustained antitryptic activity of DEW ovomucoid. These differences might be due to the different make-up of the respective ovomucoid molecules. It is well recognized that the biological activity of a protein is determined to some extent by the reactive groups in the molecule (Porter, 1953). Fraenkel-Conrat, Bean and Lineweaver (1949) have studied the composition and structure of the HEW ovomucoid in relation to its antitryptic activity, but similar information on DEW ovomucoid is not available. The present communication deals with a comparative study of the properties and chemical composition of the two purified ovomucoids. The investigations included the following: (i) determination of some chemical and physicochemical properties; (ii) identification of the constituent amino acids and sugars; (iii) estimation of carbohydrates and some amino acids with functional groups, and (iv) influence of pH on the stability of the ovomucoids to high temperatures.

EXPERIMENTAL.

Isolation of ovomucoids.—The ovomucoids were separated from the hen and duck egg whites and purified by the procedure described by the authors earlier (Narasinga Rao and Patwardhan, *loc. cit.*). Electrophoretically homogenous samples were used in the experiments described below. The sample of purified DEW ovomucoid corresponded to the fraction D₁ mentioned in the earlier publication.

Physicochemical determinations.—The following physicochemical determinations were carried out: (i) *molecular weight* was determined by measuring the osmotic pressure of a 1.0 per cent solution of the ovomucoid in 0.1 M sodium chloride according to the method of Bull (1941) using an apparatus similar to the one described by Bull and Currie (1946). (ii) *Optical rotation* of a 5 per cent solution of ovomucoid in water. (iii) *Viscosity* was measured in 0.05 M acetate buffer (pH 3.9) containing 0.1 M sodium chloride in an Ostwald viscosimeter at 25°C. as described by Fredericq and Deutsch (1949). The effect of heat and urea denaturation on the viscosity was also determined. (iv) *Isoelectric point* was determined by measuring the electrophoretic mobility of ovomucoids on paper with buffers of different pH (acetate buffers with pH range from 3.8 to 5.0 at intervals of 0.2, with ionic strength 0.1). The technique of paper electrophoresis was the same as described by Narasinga Rao and Patwardhan (*loc. cit.*). (v) *Titration curves.* The titration of the purified ovomucoids was carried out according to Cannan *et al.* (1942) between pH 1.5 and pH 8.5 using a potentiometer with quinhydrone electrode. The pH of a series of solutions (each 10 c.c.) containing 1 per cent ovomucoid (electrodialysed) and varying amounts of HCl+KCl or NaOH+KCl were determined. The total concentration of (HCl+KCl) or (NaOH+KCl) was kept constant (i.e. 0.1 N throughout). The pH of similar solutions without protein were also determined.

Amino acids and sugars in ovomucoids.—The ovomucoids were hydrolysed with 6 N HCl for 24 hours at 125°C. and the hydrochloric acid was removed by vacuum distillation. Shorter period of hydrolysis was used (eight hours) for the estimation of sulphur-containing amino acids. For sugar analysis, the ovomucoids were hydrolysed with 2 N H₂SO₄ at 100°C. for 4 hours, cooled and neutralized with

barium hydroxide. For glucosamine estimation the sulphuric acid hydrolysate was neutralized with sodium hydroxide.

The double dimension paper chromatography described by Consden *et al.* (1944) and Dent (1948) was employed for the detection of amino acids in the hydrolysates. Single dimensional chromatography with buffered solvents as described by McFarren and Mills (1952) was used to identify the leucines and phenylalanine. Paper chromatography (Patridge, 1948) and paper electrophoresis using borate buffer (Consden and Stanier, 1952) were employed for the characterization of sugars in the hydrolysates.

Quantitative determinations of mannose and glucosamine as well as of some amino acids with reactive groups (total acid groups, amino groups, amide groups, arginine, cystine, histidine, methionine, phenylalanine, tyrosine and tryptophan) were carried out. The determinations of reactive groups in the protein molecule were carried out either on the intact protein (total acid groups, amino groups, amide groups, tyrosine and tryptophan) or on its acid hydrolysate (arginine, cystine, histidine, methionine, phenylalanine) depending upon the method available. Generally, this is the method employed by other workers to determine reactive groups in a protein (Porter, *loc. cit.*; Fraenkel-Conrat *et al.*, *loc. cit.*).

Mannose was determined by the orcinol reaction of Tillman for carbohydrates as described by Rimington (1940) and Friedman (1949). Glucosamine was estimated by the method of Elson and Morgan (1933) as modified by Schlors (1951). Total acid groups which consist of carboxyl, tyrosine and -SH groups were measured using the dye-binding method of Fraenkel-Conrat and Cooper (1944). Amino groups were estimated by the ninhydrin method of Harding and MacLean (1916). Amide nitrogen was determined as described by Lewis *et al.* (1950). Arginine was determined by the Sakaguchi reaction as described by Brand and Kassell (1942). Cystine was estimated using naphthoquinone sulphonc acid method of Sullivan as described by Brown and Lewis (1941). Histidine was determined using Pauly's reaction as modified by Macpherson (1942). Methionine was estimated by the method of McCarthy and Sullivan (1941) as modified by Horn *et al.* (1946). Tyrosine was estimated using Folin's phenol reagent according to the method of Herriott (1941) in which the addition of copper sulphate was omitted. Tryptophan was estimated on intact protein using the method of Spies and Chambers (1949).

Effect of pH on the heat inactivation of ovomucoids.—The effect of heating ovomucoid solution at different pH on its antitryptic activity was studied. The latter was measured according to the method described previously (Narasinga Rao and Patwardhan, *loc. cit.*).

RESULTS AND DISCUSSION.

Precipitation and colour reactions.—Salts of heavy metals and reagents, such as trichloroacetic, glyoxalic phosphomolybdic, m-phosphoric, sulpho-salicylic and tannic acids did not precipitate either of the ovomucoids from aqueous solutions. HEW ovomucoid is precipitated at 0.78 saturation with ammonium sulphate and DEW ovomucoid between 0.45 and 0.50 saturation. It requires 2.7 volumes of alcohol to precipitate HEW ovomucoid and 2.1 volumes for the DEW counterpart. A little more than an equal volume of acetone precipitates both the

ovomucoids from solutions. Flavianic and picric acids precipitate the DEW ovomucoid but not that derived from HEW. A positive Molisch reaction is given by both indicating the presence of carbohydrate. Negative nitroprusside reaction indicated the absence of free —SH groups in both the ovomucoids.

Physicochemical properties.—The results of the determinations of the molecular weight, optical rotation and iso-electric point are given in Table I.

Molecular weight.—An average of two determinations with each sample gave a value of 37,000 for HEW ovomucoid and 38,570 for DEW ovomucoid, indicating that there is not much difference between the molecular weights of the two. The value obtained for HEW ovomucoid is higher than that reported (28,800) by Lineweaver and Murray (*loc. cit.*). The lower value reported by the latter workers may be due to the fact that they had used a crude sample of ovomucoid which is found to contain lysozyme with a low molecular weight of 17,000.

TABLE I.

Some physical constants of ovomucoids of DEW and HEW.

Physicochemical constants.	PRESENT INVESTIGATION:		Reported values for HEW ovomucoid.
	DEW ovomucoid.	HEW ovomucoid.	
Molecular weight ..	38,570	37,000	28,800 ^a , 27,000 ^b , 28,500 ^c , 49,000 ^d .
Isoelectric point -pH ₂₁ ..	4.23	4.63	4.5 ^e , 3.9 ^b , 4.3 ^f .
Optical rotation (α) _D ..	-70°.94	-57°.88	-56° ^a , -57° ^e , -65° to -70° ^h , -55° ⁱ .

a=Lineweaver and Murray, 1947. b=Fredericq and Deutsch, *loc. cit.*
 c=Lewis *et al.*, *loc. cit.* d=Mzaa, 1930. e=Hesseltvik, 1938. f=Longworth *et al.* 1940.
 g=Stacey and Woolley, 1940. h=Meyer, 1945. i=Balls and Swenson, 1934.

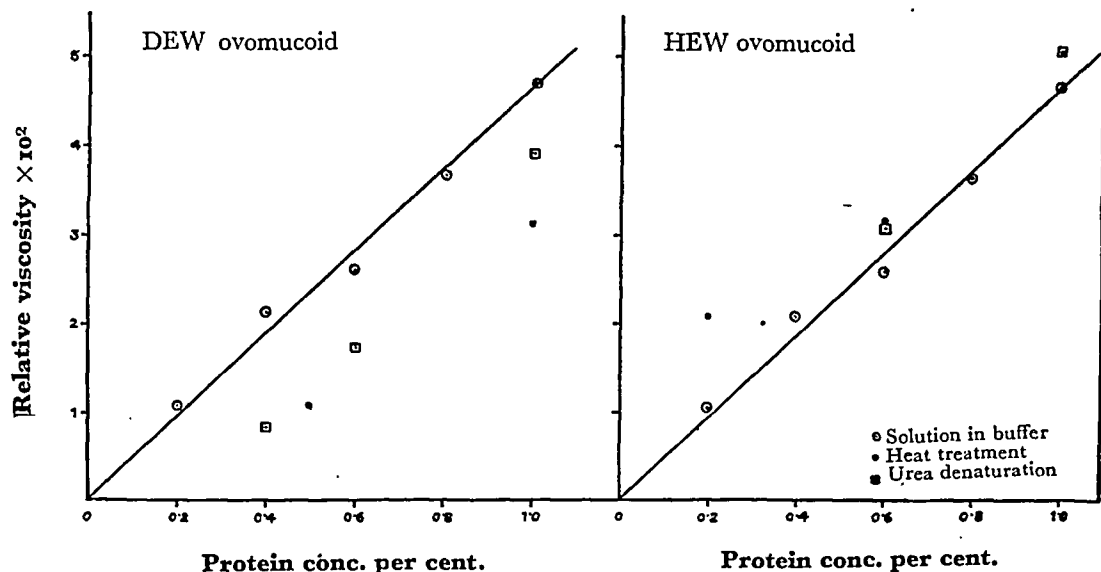
Optical rotation.—Specific rotations (α)_D²¹ for the HEW and DEW ovomucoids were found to be -57°.9 and -70°.9, respectively. The former corresponds to the reported value given in Table I. The difference in optical rotation between these two ovomucoids suggests structural differences.

Viscosity.—The results of viscosity measurements are presented in Graph 1. The relative viscosities of the two ovomucoid solutions at different concentrations were identical. This indicates that their molecular weights and shapes are similar. The effect of treatment with either urea or heat on the viscosity of the ovomucoids was very small pointing to the fact that the ovomucoid molecules are not easily denatured. Actually this treatment causes a decrease in viscosity of the DEW ovomucoid. This may be due to a decrease in the hydration of the molecule (Edsall, 1943). The results of viscosity measurements with HEW ovomucoid obtained in the present experiment agree well with those of Fredericq and Deutsch (*loc. cit.*).

Isoelectric point.—The plot of the distance moved by HEW and DEW ovomucoid spots on paper electrophoresis against pH is given in Graph 2. The

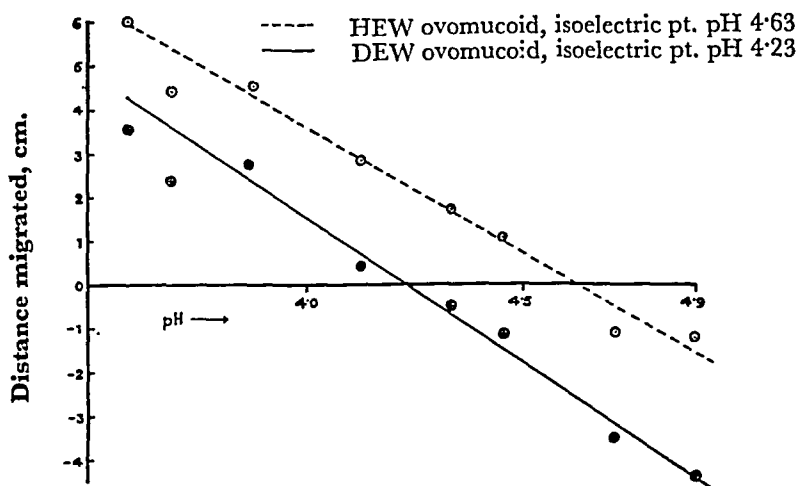
intersect of smooth curve passing through all these points and the x-axis (representing zero mobility) was taken as corresponding to the isoelectric point. The values thus arrived at were pH 4.63 and 4.23 for the HEW and DEW ovomucoids respectively (see Table I). The value of HEW ovomucoid is closer to that reported by Hesselvik (*loc. cit.*), i.e., 4.5 but is higher than pH 4.3 reported by Longsworth *et al.* (*loc. cit.*) and pH 3.9 by Fredericq and Deutsch (*loc. cit.*). The lower isoelectric point of DEW ovomucoid indicates a relatively larger proportion of free acid groups in the molecule.

GRAPH 1.



Relative viscosities of DEW and HEW ovomucoids and the effect of denaturation with heat and urea.

GRAPH 2.



Isoelectric points of DEW and HEW ovomucoids determined by paper electrophoresis.

GRAPH 3.

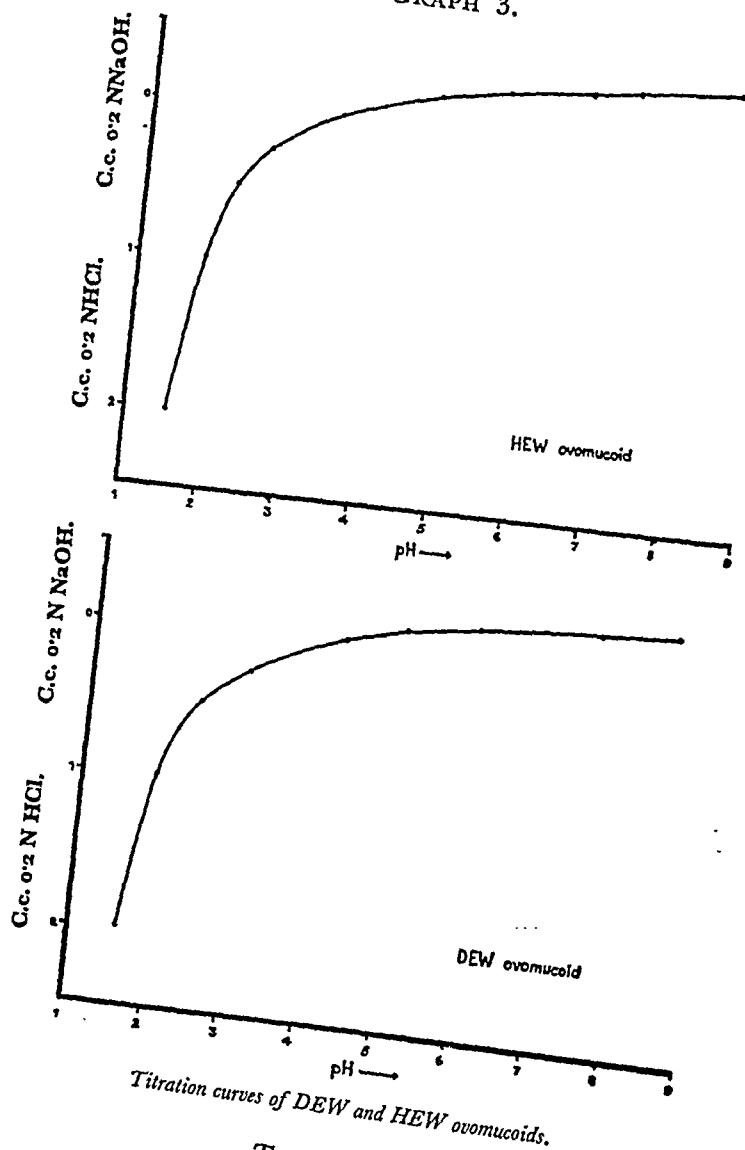


TABLE II.
Ionizable groups in DEW and HEW ovomucoids calculated from their titration curves:

Ionizable groups.	EQUIVALENTS/100 G. PROTEIN:	
	DEW ovomucoid.	HEW ovomucoid.
Total basic	0.162	0.135
Carboxyl	0.191	0.208
Imidazole	0.018	0.028

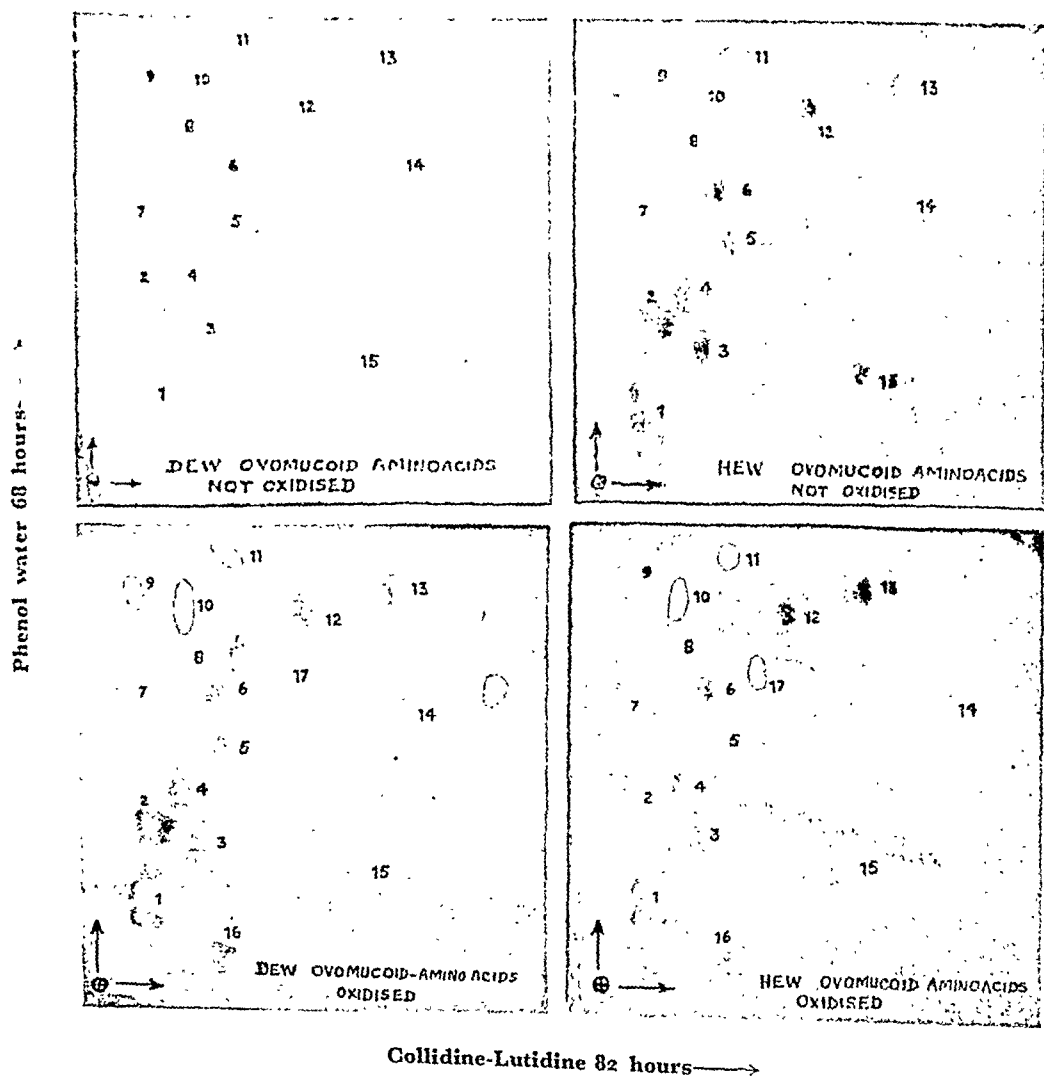
Isoionic point and ionisable groups.—The titration curves are presented in Graph 3. It is seen that the isoionic points of HEW and DEW ovomucoids in 0.1 N. KCl solution were found to be pH 3.43 and pH 4.18, respectively. The isoelectric point of HEW ovomucoid (pH 4.63) is very much different from its isoionic point, whereas the isoionic and isoelectric points of DEW ovomucoid are almost identical. Although the isoionic and isoelectric points of a protein should be theoretically identical, they are in some cases influenced by the binding of ions present in the medium by the protein molecule (Alberty, 1953). The ionizable groups in two ovomucoids calculated from titration curves are given in Table II. The titration value between isoionic point and pH 2.0 represents the total basic groups (imidazole + amino + guanidyl); that between pH 2.0 and pH 5.5 represents total carboxyl groups and that between pH 5.5 and pH 8.5 imidazole groups (Alberty, *loc. cit.*). It can be seen that DEW ovomucoid contains almost the same quantities of carboxyl groups as HEW ovomucoid, more basic groups and slightly less imidazole groups than HEW ovomucoid.

Amino acids and sugars in ovomucoids.—The chromatograms of amino acids of DEW and HEW ovomucoid hydrolysates are given in (Plate XXIV). It can be seen that the ovomucoids do not differ qualitatively from each other, the same amino acids being present in both. In general, the basic amino acid spots were much weaker than the others and the acidic amino acids were prominent. The spot due to histidine was very weak in both. However, there were certain marked differences in the quantities of certain amino acids present in the two ovomucoids as could be judged by the intensity of the spots and later confirmed by quantitative determinations. It was observed that HEW ovomucoid contained more arginine and glucosamine than DEW ovomucoid did. On the other hand, tyrosine and methionine were more in DEW ovomucoid than in HEW ovomucoid.

Among other things observed in these chromatograms, one was the appearance of a spot, which corresponded to methionine sulphoxide, near the histidine spot, even without oxidation. Methionine sulphoxide is formed most probably during the phenol run (Dent, *loc. cit.*). It was found on the chromatogram of pure amino acids and the spot intensified when methionine was incorporated in the hydrolysate. Secondly, glucosamine occupied a position on the chromatograms of both hydrolysates and the amino acid mixture, which indicated much slower movement during phenol run. It appears, therefore, that the presence of other amino acids (probably acidic amino acids) influences the R_f value of glucosamine during phenol run. But it was observed that during Collidine-Lutidine run the R_f value of glucosamine was not influenced by the presence of other amino acids.

Chromatograms and paper electrophoretic patterns of sugars in ovomucoid hydrolysates are given in Text-figures 1 and 2, respectively.

It can be seen that in both the cases only mannose and glucosamine were detected. This fact was confirmed by the two dimensional chromatography. Galactose could not be detected; incorporation of galactose gave rise to a separate third spot. These observations establish clearly that both the ovomucoids contain only mannose and glucosamine and no galactose. This finding does not substantiate the claim of Sørensen (1934) and of Stacey and Woolley (*loc. cit.*) that HEW ovomucoid contains galactose and further confirms the failure of other workers



Two adimensional chromatograms of amino acids in hydrolysates of DEW and HEW ovomucoids with and without oxidation.

- | | | |
|---|------------------------|-------------------------|
| 1. Aspartic acid | 2. Glutamic acid | 3. Serine |
| 4. Glycine | 5. Threonine | 6. Alanine |
| 7. Lysine | 8. Histidine | 9. Arginine |
| 10. Methionine sulphoxide | 11. Proline | 12. Valine |
| 13. Penyl-alanine, Leucine, Isoleucine & Methionine | | 14. Tyrosine |
| 15. Glucosamine | 16. Cystine (oxidised) | 17. Methionine sulphone |